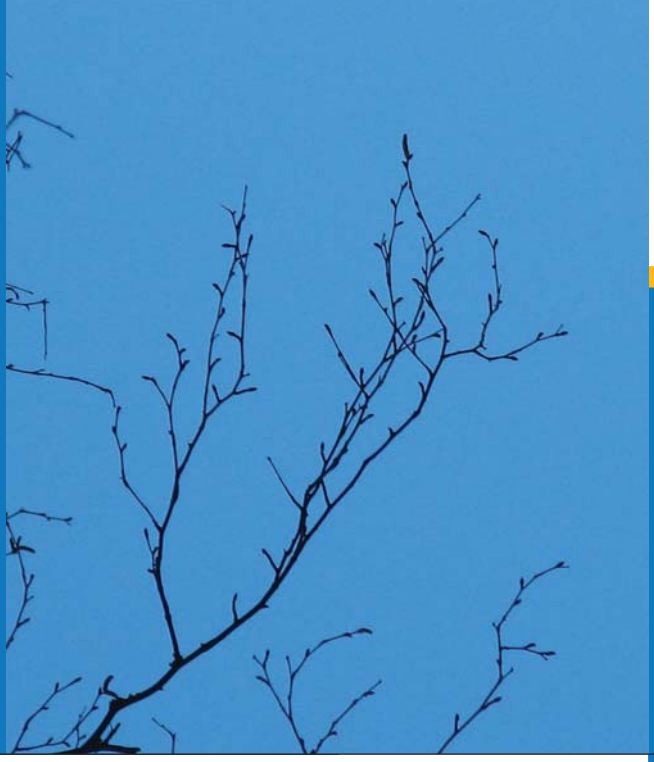

Carita Savolainen-Kopra

Molecular Epidemiology of Human Rhinoviruses

Publications of the National Public Health Institute A 2/2006

Department of Viral Diseases and Immunology
National Public Health Institute
Helsinki, Finland
and
Department of Biological and Environmental Sciences
Faculty of Biosciences
University of Helsinki

Carita Savolainen-Kopra

MOLECULAR EPIDEMIOLOGY OF HUMAN RHINOVIRUSES

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Biosciences,
University of Helsinki,
for public examination in Auditorium 1041, Biocenter 2, Viikinkaari 5, Helsinki,
on March 17, 2006, at 12 noon.

Department of Viral Diseases and Immunology, National Public Health Institute
Helsinki, Finland
and
Department of Biological and Environmental Sciences
Faculty of Biosciences, University of Helsinki,
Helsinki, Finland

Helsinki 2006

Publications of the National Public Health Institute
KTL A2 / 2006

Julkaisija-Utgivare-Publisher

Kansanterveyslaitos (KTL)
Mannerheimintie 166
00300 Helsinki
Puh. vaihde (09) 474 41, telefax (09) 4744 8408

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsingfors
Tel. växel (09) 474 41, telefax (09) 4744 8408

National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki, Finland
Telephone: +358 9 474 41, Fax: +358 9 4744 8408

ISBN 951-740-602-9
ISSN 0359-3584
ISBN 951-740-603-7 (pdf)
ISSN 1458-6290 (pdf)

Kannen kuva - Cover graphic Carita Savolainen-Kopra

Edita Prima Oy
Helsinki 2006

Supervised by

Professor Tapani Hovi
Department of Viral Diseases and Immunology
National Public Health Institute
Helsinki, Finland

Reviewed by

Professor Timo Hyypiä
Department of Virology
University of Turku,
Turku, Finland

Docent Alexander Plyusnin
Department of Virology
University of Helsinki,
Helsinki, Finland

Opponent

Professor Michael Lindberg
Department of Chemistry and Biomedical Sciences
University of Kalmar,
Kalmar, Sweden

To Marcus and Christa

Carita Savolainen-Kopra, Molecular Epidemiology of Human Rhinoviruses
Publications of the National Public Health Insitute, A2/2006, 86 Pages
ISBN 951-740-602-9; 951-740-603-7 (pdf-version)
ISSN 0359-3584; 1458-6290 (pdf-version)
http://www.ktl.fi/portal/4043

ABSTRACT

The first part of this work investigates the molecular epidemiology of a human enterovirus (HEV), echovirus 30 (E-30). This project is part of a series of studies performed in our research team analyzing the molecular epidemiology of HEV-B viruses. A total of 129 virus strains had been isolated in different parts of Europe. The sequence analysis was performed in three different genomic regions: 420 nucleotides (nt) in the VP4/VP2 capsid protein coding region, the entire VP1 capsid protein coding gene of 876 nt, and 150 nt in the VP1/2A junction region. The analysis revealed a succession of dominant sublineages within a major genotype. The temporally earlier genotypes had been replaced by a genetically homogenous lineage that has been circulating in Europe since the late 1970s. The same genotype was found by other research groups in North America and Australia. Globally, other cocirculating genetic lineages also exist. The prevalence of a dominant genotype makes E-30 different from other previously studied HEVs, such as polioviruses and coxsackieviruses B4 and B5, for which several coexisting genetic lineages have been reported.

The second part of this work deals with molecular epidemiology of human rhinoviruses (HRVs). A total of 61 field isolates were studied in the 420-nt stretch in the capsid coding region of VP4/VP2. The isolates were collected from children under two years of age in Tampere, Finland. Sequences from the clinical isolates clustered in the two previously known phylogenetic clades. Seasonal clustering was found. Also, several distinct serotype-like clusters were found to co-circulate during the same epidemic season. Reappearance of a cluster after disappearing for a season was observed. The molecular epidemiology of the analyzed strains turned out to be complex, and we decided to continue our studies of HRV.

Only five previously published complete genome sequences of HRV prototype strains were available for analysis. Therefore, all designated HRV prototype strains (n=102) were sequenced in the VP4/VP2 region, and the possibility of genetic typing of HRV was evaluated. Seventy-six of the 102 prototype strains clustered in HRV genetic group A (HRV-A) and 25 in group B (HRV-B). Serotype 87 clustered separately from other HRVs with HEV species D. The field strains of HRV represented as many as 19 different genotypes, as judged with an approximate demarcation of a 20% nt difference in the VP4/VP2 region.

The interserotypic differences of HRV were generally similar to those reported between different HEV serotypes (i.e. about 20%), but smaller differences, less than 10%, were also observed. Because some HRV serotypes are genetically so closely related, we suggest that the genetic typing be performed using the criterion "the closest prototype strain". This study is the first systematic genetic characterization of all known HRV prototype strains, providing a further taxonomic proposal for classification of HRV. We proposed to divide the genus *Human rhinoviruses* into *HRV-A* and *HRV-B*.

The final part of the work comprises a phylogenetic analysis of a subset (48) of HRV prototype strains and field isolates (12) in the nonstructural part of the genome coding for the RNA-dependent RNA polymerase (3D). The proposed division of the HRV strains in the species HRV-A and HRV-B was also supported by 3D region. HRV-B clustered closer to HEV species B, C, and also to polioviruses than to HRV-A. Intraspecies variation within both HRV-A and HRV-B was greater in the 3D coding region than in the VP4/VP2 coding region, in contrast to HEV. Moreover, the diversity of HRV in 3D exceeded that of HEV. One group of HRV-A, designated HRV-A', formed a separate cluster outside other HRV-A in the 3D region. It formed a cluster also in the capsid region, but located within HRV-A. This may reflect a different evolutionary history of distinct genomic regions among HRV-A. Furthermore, the tree topology within HRV-A in the 3D region differed from that in the VP4/VP2, suggesting possible recombination events in the evolution of the strains. No conflicting phylogenies were observed in any of the 12 field isolates. Possible recombination was further studied using the Similarity and Bootscanning analyses of the complete genome sequences of HRV available in public databases. Evidence for recombination among HRV-A was found, as HRV2 and HRV39 showed higher similarity in the nonstructural part of the genome. Whether HRV2 and HRV39 strains – and perhaps also some other HRV-A strains not yet completely sequenced – are recombinants remains to be determined.

Keywords: molecular epidemiology, human rhinovirus, human enterovirus, echovirus

Carita Savolainen-Kopra, Molecular Epidemiology of Human Rhinoviruses
Kansanterveyslaitoksen julkaisuja, A2/2006, 86 sivua
ISBN 951-740-602-9; 951-740-603-7 (pdf-versio)
ISSN 0359-3584; 1458-6290 (pdf-versio)
http://www.ktl.fi/portal/4043

TIIVISTELMÄ

Työn ensimmäinen osa koostuu molekyyliepidemiologisesta tutkimuksesta, jossa tutkittiin erään ihmisen enteroviruksen (HEV), echovirus 30:n kliinisiä isolaatteja. Tämä tutkimus on osa tutkimusryhmämme toteuttamaa HEV-B virusten molekyyliepidemiologiaa tutkivaa sarjaa. Tutkimuksessa mukana olleet kaikkiaan 129 viruskantaa oli eristetty eri puolella Eurooppaa. Sekvenssianalyysi käsitti kolme erillistä genomialuetta; 420 nukleotidin pituisen VP4/VP2-kapsidiproteiineja koodaavan osan, koko 876 nukleotidin pituisen VP1-kapsidiproteiinia koodaavan geenin sekä 150 nukleotidin pituisen VP1/2A- risteysalueen. Analyysi paljasti vallitsevien geneettisten alaryhmien jatkumon yhden päägenotyypin sisällä. Aiemmat genotyypit olivat korvautuneet geneettisesti yhtenäisellä alatyypillä, joka oli kiertänyt Euroopassa 1970-luvun lopulta lähtien. Muut tutkimusryhmät olivat havainneet saman genotyypin myös Pohjois-Amerikassa ja Australiassa. Kuitenkin maailmanlaajuisesti muitakin yhtä aikaa kiertäviä genotyyppejä on havaittu. Echovirus 30:lla havaittu yhden päägenotyypin vallitsevuus eroaa muista enterovirusserotyypeistä, joita on tutkittu molekyyliepidemiologian keinoin. Tällaisia ovat esimerkiksi poliovirukset sekä coxsackievirukset B4 ja B5, joilla on havaittu useita samanaikaisia geneettisesti eroavia alatyyppejä.

Työn toisessa osassa ihmisen rinovirusten molekyyliepidemiologiaa tutkittiin 61 kliinisen isolaatin geneettisellä analyysillä 420 nukleotidin pituisella VP4/VP2-kapsidiproteiinialueella. Virusisolaatit oli kerätty alle kaksivuotiaista lapsista Tampereen alueella. Kliinisistä isolaateista saadut sekvenssit jakautuivat kahteen ennalta tunnettuun fylogeneettiseen pääryhmään. Alaryhmiä muodostui vuodenaikaisvaihtelun mukaan. Myös saman epidemiakauden aikana havaittiin kiertäneen useita erillisiä serotyypin kaltaisia klustereita. Lisäksi, klusterin havaittiin ilmestyneen uudelleen yhden epidemiakauden poissaolon jälkeen. Analysoitujen rinoviruskantojen molekyyliepidemiologia näytti monimutkaiselta, joten päätimme jatkaa rinovirustutkimuksia.

Rinovirusten sekvenssianalyysiin oli saatavilla vain viisi aiemmin kokonaan sekvensoitua prototyyppikantaa. Sen vuoksi kaikki nimetyt 102 rinovirusten prototyyppikantaa sekvensoitiin VP4/VP2-alueelta ja mahdollisuutta rinovirusten geneettiseen tyypitykseen tutkittiin. 76 prototyyppikantaa klusteroitui rinovirusten geneettiseen ryhmään A (HRV-A) ja 25 ryhmään B (HRV-B). Serotyyppi 87 erosi muista rinoviruksista ja klusteroitui enterovirus D- ryhmään. Rinovirusten kliiniset isolaatit edustivat 19 erillistä serotyyppiä, kun kriteerinä käytettiin 20% eroavuutta nukleotidisekvenssissä VP4/VP2-alueella. Serotyyppien väliset erot rinoviruksilla olivat yleensä samaa luokkaa kuin enteroviruksilla on havaittu (noin 20%), mutta myös pienempiä, alle 10%, eroja havaittiin. Koska osa rinovirusserotyypeistä on geneettisesti hyvin lähellä toisiaan, ehdotamme, että geneettisen tyypityksen kriteerinä käytetään ”lähintä prototyyppiä”. Tämä tutkimus oli ensimmäinen kaikkien tunnettujen rinovirusprototyyppikantojen systemaattinen geneettinen kartoitus ja se tarjoaa pohjan ihmisen rinovirusten taksonomiselle luokittelulle kahteen ryhmään *HRV-A* ja *HRV-B*.

Työn viimeinen osa käsittelee fylogeneettistä analyysia, jossa oli mukana 48 rinovirusten prototyyppikantaa sekä 12 kliinistä isolaattia. Tutkittavana genomialueena oli ei-strukturaalinen viruksen RNA polymeraasia koodaava 3D-alue. Rinoviruskannat jakautuivat aiemmin määriteltyihin ryhmiin HRV-A ja HRV-B myös 3D-alueella. HRV-B klusteroitui geneettisesti lähemmäs enterovirus-B, -C ja poliovirusryhmiä kuin HRV-A:ta. Ryhmien sisäinen variaatio sekä HRV-A:ssa että HRV-B:ssä oli suurempaa 3D- kuin VP4/VP2-kapsidialueella, toisin kuin enteroviruksilla. Lisäksi rinovirusten variaatio 3D:ssa oli suurempaa kuin enteroviruksilla. 3D-alueella havaittiin erillinen klusteri, joka kapsidialueella kuului HRV-A:han. Se nimettiin HRV-A’:ksi. Tämä havainto saattaa olla seurausta HRV-A rinovirusten eri genomialueiden erilaisesta evoluutiohistoriasta. Myös HRV-A:n fylogeneettisten puiden topologiassa havaittiin eroja kapsidialueen ja ei-strukturaalialueen välillä, mikä saattaa viitata eri kantojen rekombinaatioon. Kuitenkin kaikki 12 tutkittua kliinistä isolaattia klusteroituivat samoin kuin kapsidialueella. Mahdollista rekombinaatiota selvitettiin julkisissa tietokannoissa saatavissa olevien rinovirusten kokogenomisekvenssien Similarity ja Bootscanning –analyyseillä. Todisteita rekombinaatiosta HRV-A:ssa saatiin, kun HRV2 ja HRV39 osoittivat keskimääräistä suurempaa samankaltaisuutta genomin ei-strukturaaliosassa. Ovatko juuri HRV2 ja HRV39 rekombinoituneita kantoja vai kenties jotkut muut toistaiseksi sekvensoimattomat HRV-A serotyypit, jää vielä selvitettäväksi.

Avainsanat: Molekyyliepidemiologia, ihmisen rinovirus, ihmisen enterovirus, echovirus

CONTENTS

ABBREVIATIONS

ATCC	American Type Culture Collection
AOM	Acute otitis media
cDNA	Complementary DNA
CDC	Centers for Disease Control and Prevention, Atlanta, USA
CPE	Cytopathic effect
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
FinOM	Finnish Otitis Media Study
GCG	Genetics Computer Group, Inc., USA
HEV	Human enterovirus
HI	Haartman Institute, University of Helsinki, Finland
HKY85	Haseqawa, Kishino, and Yano model
HRV	Human rhinovirus
ICAM	Intercellular adhesion molecule
IRES	Internal ribosome entry site
K2P	Kimura two-parameter model
LDL	Low-density lipoprotein
MEF	Middle ear fluid
ML	Maximum likelihood
NCR	Noncoding region
NIAID	National Institute of Allergy and Infectious Diseases
NIm	Neutralizing immunogenic site
NJ	Neighbor-joining
NPA	Nasopharyngeal aspirate
nt	Nucleotide

OPV	Oral poliovirus vaccine
PV	Poliovirus
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RFLP	Restriction fragment length polymorphism
RIVM	National Institute for Public Health and the Environment, Bilthoven, The Netherlands
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
TCID	Tissue culture infectious dose
TS/TV	Transition/transversion ratio
UPGMA	Unweighted Pair Group Arithmetic Mean
VLDL	Very low-density lipoprotein
VP	Viral protein
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Savolainen, C., Hovi, T., and Mulders, M.N. 2001. Molecular epidemiology of echovirus 30 in Europe: succession of dominant sublineages within a single major genotype. Archives of Virology 146: 521-537.

II Savolainen, C., Mulders, M.N., and Hovi, T. 2002. Phylogenetic analysis of rhinovirus isolates collected during successive epidemic seasons. Virus Research 85: 41-46.

III Savolainen, C., Blomqvist, S., Mulders, M.N., and Hovi, T. 2002. Genetic clustering of all 102 human rhinovirus prototype strains: serotype 87 is close to human enterovirus 70. Journal of General Virology 83: 333-340.

IV Savolainen, C., Laine, P., Mulders, M.N., and Hovi, T. 2004. Sequence analysis of human rhinoviruses in the RNA-dependent RNA polymerase coding region reveals large within-species variation. Journal of General Virology 85: 2271-2277.

Some unpublished material is also presented.

1 INTRODUCTION

The most frequent acute illness in humans worldwide is acute respiratory infection. The frequent form of it, common cold, is predominantly caused by human rhinoviruses (HRVs) (Arruda et al. 1997; Mäkelä et al. 1998). Identification of HRV is usually based on detection of viral genome by RT-PCR. This is mainly because of the laborious isolation procedure and the large number of HRV serotypes. Despite the common nature of HRV, the laboratory diagnosis of this virus group is generally restricted to a genus level of identification and serotyping is not generally performed. Thus, very little is known about the incidence and characteristics of different HRV serotypes. The genetic features and relationships of a closely related genus, enteroviruses, have been extensively studied in recent years. The novel genetic typing of enteroviruses (Oberste et al. 1999b) based on the sequence in the VP1 coding part of the genome has expanded the possibilities of enterovirus research. As a result, several new enterovirus types have been identified.

Development of antiviral drugs requires information on the molecular features of the target group of viruses. HRVs have been recognized as a major cause of an economically important disease. As they comprise more than 100 serotypes, new information on the genetic relationships of these viruses would be extremely important. Furthermore, information on currently circulating HRV strains is very limited and needs updating.

2 REVIEW OF THE LITERATURE

2.1 HUMAN PICORNAVIRUSES

2.1.1 GENERAL ASPECTS

Picornaviruses are among the smallest RNA-containing animal viruses known. They comprise one of the largest and most important virus families of human and veterinary pathogens, *Picornaviridae*. Well known members of picornaviruses include polioviruses, human hepatitis A virus, and foot-and-mouth disease virus. Headway made in investigating, for example, poliomyelitis and foot-and-mouth disease, both of which are medically and economically significant, has contributed greatly to the development of modern virology.

2.1.2 STRUCTURE

Picornaviruses are nonenveloped particles of about 30 nm in diameter. They possess an icosahedral capsid containing 60 copies of each of the four capsid proteins (VP1-4). The capsid is composed of 12 pentagon-shaped pentamers of five protomers, each holding one copy of four structural proteins. The major capsid proteins, VP1 to VP3, are folded into eight-stranded antiparallel β-sheets with a jelly-roll topology. The β-barrels of VP1 proteins are located around a fivefold axis of symmetry, while VP2 and VP3 are located around the threefold axis. VP4, the smallest structural protein, is located on the inner surface of the capsid. At the fivefold axis, there is a star-shaped plateau surrounded by a circular canyon. The canyon outlines a protrusion of five copies of VP1 from the surrounding VP2 and VP3. Beneath the canyon floor, within the core of VP1, is a hydrophobic tunnel, a “pocket”. (Racaniello 2001).

2.1.3 GENOMIC STRUCTURE AND EXPRESSION

The viral genome is a single-stranded, messenger-sense RNA of 7000-8800 nt with a single open reading frame (Fig. 1). The basic structure is shared by all picornaviruses. There is a short peptide (VPg or 3B) covalently coupled to the 5’ end of the RNA. The 5’ noncoding region (NCR) is involved in the initiation of translation, directing

ribosomes into the internal ribosome entry site (IRES). The NCR is followed by the protein coding region. It encodes a single polyprotein, which is proteolytically cleaved into precursor proteins P1, P2, and P3, and thereafter into structural proteins VP1 to VP4 (P1 region) and seven nonstructural proteins (P2 and P3). The nonstructural proteins include viral proteases (2A, 3C, and 3CD) and the RNA-dependent RNA polymerase (3D). In addition, 2C is a helicase, and 2B, 2BC, 3A and 3AB are associated with various functions in the replication of the viral RNA. These regions are followed by a short 3'NCR and a poly-A tail. The 3'NCR has a role in initiating the synthesis of negative-strand RNA. (Racaniello 2001).

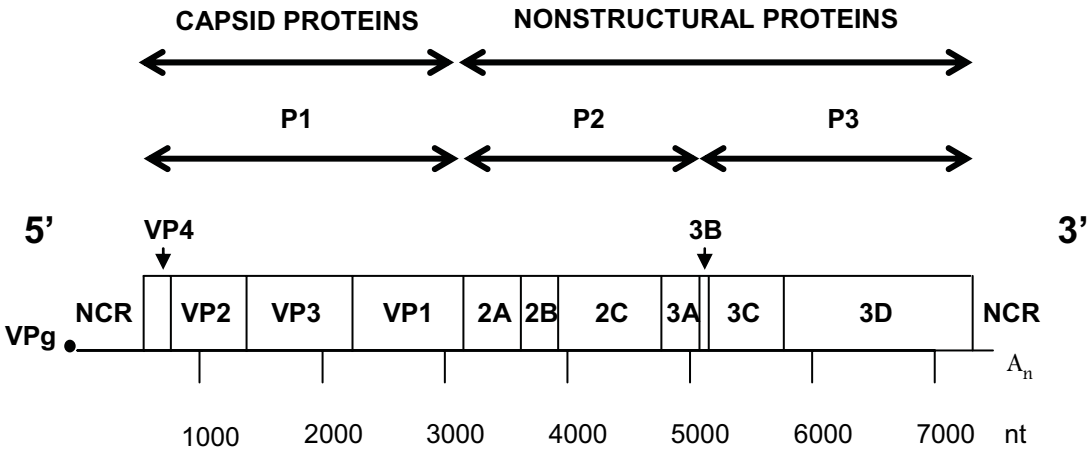

Figure 1. The *Picornavirus* genome.

HRVs infect cells of the nasopharyngeal region i*n vivo*. Replication takes place in the cytoplasm of the host cell. In order to enter the cell, viruses attach to a cell surface receptor. Rhinoviruses use either intercellular adhesion molecule 1 (ICAM-1) (major receptor group) (Greve et al. 1989; Staunton et al. 1989; Tomassini et al. 1989) or a member of the low-density lipoprotein (LDL) receptor family (minor receptor group) (Hofer et al. 1994; Gruenberger et al. 1995; Marlovits et al. 1998). ICAM-1 is a member of the immunoglobulin superfamily. Cryoelectron microscopy and image reconstruction analysis for HRV3 (Xing et al. 2003), HRV14 (Kolatkar et al. 1999), and HRV16 (Olson et al. 1993) have revealed that the binding site of ICAM-1 is at the base of the canyon. The LDL receptor family includes the LDL receptor itself, the very low-density lipoprotein (VLDL) receptor and the LDL-receptor-related protein. They act as mediators in the transport of lipoproteins into the cells by receptor-mediated endocytosis. The VLDL receptor footprint, located at the star-shaped plateau of the icosahedral fivefold axis, is formed by the BC and HI loops of VP1 (Hewat et al. 2000).

Upon attachment to the cellular receptors, conformational changes in the viral capsid are triggered. The uncoating process leads to a release of viral RNA into the cytoplasm. The major and minor receptor group viruses are proposed to have different mechanisms for uncoating. HRV14, binding to ICAM-1, rapidly uncoats without the participation of a cellular machinery (Greve et al. 1991; Casasnovas and Springer 1994). Release of HRV2 RNA, in contrast, is mediated by clathrin-dependent internalization of the virus into acidic endosomal compartments (Neubauer et al. 1987; Prchla et al. 1994; Schober et al. 1998; Snyers et al. 2003).

The viral RNA is translated in the cytoplasm by exploiting the cellular machinery. The viral proteins are synthesized first to obtain the RNA-dependent RNA polymerase that previously was nonexistent in the cell. The complete viral protein coding region is translated into a long polyprotein precursor, which is co-translationally cleaved into the intermediates P1, P2, and P3 by two proteinases, 2A and 3C/3CD. P2 and P3 proteins are cleaved into seven nonstructural proteins in addition to several intermediates acting in RNA replication and polyprotein processing. The viral RNA is then used as a template for synthesis of negative-strand RNA by polymerase 3D. This strand is used in the replication complex in the synthesis of positive-strand copies, which in turn are translated into viral proteins or encapsidated into the assembling virions. The assembly of new virions begins with P1 cleavage into VP0, VP1, and VP3.

VP0 is further cleaved to VP4 and VP2 in the process called maturation cleavage. The new infectious virions are assembled and released from the cell. (Racaniello 2001).

One replication cycle of rhinoviruses is typically completed in 10-12 hours, but depending on the type of virus can take up to 17 hours. New virus progeny, generally detected in 5-7 hours, can appear as late as 9 hours. (Stott and Killington 1972).

2.1.4 TAXONOMY AND SUBGROUPING

The family *Picornaviridae* contains nine genera according to the most recent taxonomy (Stanway et al. 2005). These genera are: *Enterovirus, Rhinovirus, Cardiovirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus, and Teschovirus* (Fig. 2). Each genus is further divided into species containing one or more antigenically distinct serotypes. Currently the total number of serotypes in *Picornaviridae* is well beyond 200. The taxonomy of *Picornaviridae* is changing and new tentative species as well as new, partially characterized serotypes have been proposed to be assigned to the family (Stanway et al. 2005).

Figure 2. Neighbor-joining tree depicting genetic relationships of the family *Picornaviridae* in the capsid protein coding region. Numbers indicate Bootstrap values (1000 replicates). LV, *Ljungan virus*; HPeV, *Human parechovirus*, BKV, *Bovine kobuvirus*; AIV, *Aichi virus*; PEV-A, *Porcine enterovirus A*; HEV-B, *Human enterovirus B*, PV, *Poliovirus*; HEV-C, *Human enterovirus C*; HRV-A, *Human rhinovirus A*, HRV-B, *Human rhinovirus B*, SEV-A, *Simian enterovirus A*, HEV-A, *Human enterovirus A*, BEV, *Bovine enterovirus*; PEV-B, *Porcine enterovirus B*; HEV-D, *Human enterovirus D*; PTV, *Porcine teschovirus*, EMCV, *Encephalomyocarditis virus*, ThV, *Theilovirus*, ERBV, *Equine rhinitis B virus*, FMDV, *Foot-and-mouth disease virus*; ERAV, *Equine rhinitis A virus*; HAV, *Hepatitis A virus*; AELV, *Avian encephalomyelitis-like virus*. Reprinted from Stanway et al. (2005) with permission from Elsevier. Only in printed version.

2.1.4.1 CLASSIFICATION OF ENTEROVIRUSES

The traditional classification of HEVs is based on shared biological properties, i.e. hosts and pathogenicity in experimental animals. The official classification comprises 68 serotypes (Stanway et al. 2005). However, the enterovirus genus currently consists of more than 100 separate members, several of which have not been officially classified yet. The new serotypes have been assigned based on genetic features in the VP1 capsid protein coding gene. HEVs are further divided into the species HEV-A to HEV-D. Polioviruses (PVs) are included in HEV-C (Table 1).

Table 1. Members of the *Enterovirus* genus

Species (number of serotypes)	Representatives
Human enterovirus A (13)	Human coxsackieviruses (CV) A2-A8, A10, A12, A14, A16 Human enterovirus (EV) 71, 76
Human enterovirus B (41)	Human coxsackievirus A9, B1-6 Human echovirus (E) 1-7, 9, 11-21, 24-27, 29-33 Human enterovirus 69, 73-75, 77, 78
Human enterovirus C (9) + poliovirus (3)	Human coxsackieviruses A1, A11, A13, A17, A19-22, A24, Human polioviruses (PV) 1-3
Human enterovirus D (2)	Human enterovirus 68, 70
unclassified enteroviruses (> 50)	

Human echo (*E*nteric *C*ytopathogenic *H*uman *O*rphan) –viruses were thus named, since after the first isolations their relationship to human disease was unknown, and they also failed to produce illness in laboratory animals (Committee on the ECHO Viruses 1955). They have subsequently been classified into HEV-B (Table 1).

2.1.4.2 SUBGROUPING OF HUMAN RHINOVIRUSES

2.1.4.2.1 CELL GROWTH

In early studies, the rhinovirus strains were classified as H or M depending on their growth properties in different cell lines (Taylor-Robinson and Tyrrell 1962; Hilleman 1967). H strains are able to replicate only in human embryonic kidney cells, while M strains grow in both human and monkey kidney cells. The separation based on growth properties has subsequently been inaccurate, with exceptions found in both classes (Mufson et al. 1965; Phillips et al. 1965; Douglas et al. 1966).

2.1.4.2.2 RECEPTOR SPECIFICITY

The first classification of rhinoviruses into two receptor groups was based on competitive binding assays between pairs of serotypes before the receptors were known (Abraham and Colonno 1984). When the ICAM-1 was identified as a major receptor, 101 rhinovirus serotypes were divided into receptor groups (Uncapher et al. 1991). A total of 91 serotypes belonged to the major group, while 9 serotypes (HRV1 subtypes A and B, HRV2, HRV29, HRV30, HRV31, HRV44, HRV47, HRV49, and HRV62) were members of the LDL-receptor-using minor group. HRV87 could not be classified into either of these groups. Its attachment to HeLa cells was inhibited by pretreatment with neuraminidase, and thus, it was suggested that this virus uses sialic acid as a receptor (Uncapher et al. 1991). Confirmation of the receptor groups was achieved when inhibitory activities of soluble ICAM-1 molecules were tested against all rhinoviruses (Crump et al. 1993). All major-group rhinoviruses, except HRV23 and HRV25, were inhibited by soluble ICAM-1. Neither the replication of the minor-group viruses nor that of HRV87 was affected. Recently, HRV23 and HRV25 have been shown to belong to the minor receptor group (Vlasak et al. 2005). Furthermore, the ICAM-1-using serotypes have been demonstrated to have preference over the natural variants of the molecule (Xiao et al. 2004). HRV14 binds to ICAM-1 only transiently but forms a stable complex with a variant ICAM-1^{Kilifi}, whereas HRV16 does not bind to ICAM-1^{Kilifi}.

2.1.4.2.3 SENSITIVITY TO ANTIVIRAL AGENTS

Considerable efforts have been directed at developing antiviral therapy against rhinovirus infection. Over the course of these studies, several structurally unrelated antiviral compounds have been shown to inhibit replication of rhinoviruses. The binding site for most of the antiviral agents is a hydrophobic pocket in the capsid protein VP1. A systematic, multivariate analysis of 100 rhinovirus serotypes tested against a panel of 15 antiviral compounds divided rhinoviruses into two antiviral groups. Antiviral group A included 33 serotypes with a greater than average susceptibility to elongated antiviral compounds such as WIN51711. Antiviral group B contained 67 serotypes susceptible to structurally shorter antiviral agents, including chalcone, dichloroflavan, and R61837. (Andries et al. 1990; Andries et al. 1991).

2.1.4.2.4 ANTIGENIC DIFFERENCES

A systematic analysis of antigenic relationships of 90 rhinovirus serotypes revealed that 50 of these serotypes could be divided to 16 antigenic groups (Cooney et al. 1982). Each of these groups contained 2-10 antigenically related serotypes. Reciprocal neutralization was observed for virus pairs HRV1A-HRV1B, HRV2-HRV49, HRV3-HRV14, HRV9-HRV32, HRV12-HRV78, HRV13-HRV41, HRV15-HRV74, HRV29-HRV44, and HRV36-HRV58 (Cooney et al. 1973; Schieble et al. 1974; Cooney et al. 1982). One-way cross-reactions have been reported for pairs HRV5-HRV42, HRV6-HRV14, HRV9-HRV67, HRV11-HRV40, HRV11-HRV74, HRV17-HRV42, HRV17-HRV70, HRV22-HRV61, HRV32-HRV67, HRV36-HRV50, HRV36-HRV89, HRV39-HRV54, HRV40-HRV56, HRV60-HRV38, HRV66-HRV77, and HRV76-HRV11 (Cooney et al. 1973; Calhoun et al. 1974; Cooney et al. 1982). Heterotypic antibody responses have also been demonstrated in humans after natural infection. Natural infection with one of the related serotypes (HRV1A and HRV1B, HRV2 and HRV49, HRV23 and HRV30 or HRV29, and HRV44 and HRV62) raised antibodies also against another virus and was suggested to provide cross-protection (Mogabgab et al. 1975).

2.1.4.2.5 GENETIC RELATIONSHIPS

A complete genome sequence of a HRV was first published in 1984 for HRV14 (Stanway et al. 1984) and one year later for HRV2 (Skern et al. 1985) (Table 3). The first study on the genetic relationships of HRV was published in 1986 by (Al-Nakib et al. 1986) and colleagues. This study was based on cDNA hybridization. Of the 54 serotypes investigated, HRV3, HRV4, HRV17 and HRV72 were suggested to be the closest relatives of HRV14. The next complete genome sequence was published for HRV89, after which the molecular relationships of the three known complete genome sequences of HRV were compared (Duechler et al. 1987). Extensive similarity between HRV89 and HRV2 was found, while HRV14 appeared to be more distant. The division into two genetic clades was corroborated in comparisons of partial 5'NCR of an additional 10 prototype strains (Mori and Clewley 1994). Altogether 39 HRV serotypes were analyzed in the 5'NCR and clustering into two major clades confirmed (Andeweg et al. 1999). Furthermore, HRV87 was found to be the most divergent, forming a clade of its own. The first wider study on genetic relationships of HRV in the capsid coding region was published in 1995 by Horsnell and colleagues. This study dealt with partial sequences in the VP2 capsid protein coding region, including the immunogenic site NImII, of 21 HRV prototype strains. The two major genetic clades were substantiated. Eighteen of the 21 sequenced serotypes were shown to belong to the HRV2-related group, while three serotypes formed the HRV14-related group. Thus far, eight complete HRV genomes have been sequenced (Table 2).

Table 2. Human rhinovirus (HRV) prototype strains for which a complete genome sequence has been published.

HRV prototype strain	Reference
HRV1B	Hughes et al. 1988
HRV2	Skern et al. 1985
HRV9	Leckie 1987
HRV14	Stanway et al. 1984
HRV16	Lee et al. 1995
HRV39	Harris and Racaniello 2005
HRV85	Stanway 1989
HRV89	Duechler et al. 1987

2.1.5 EVOLUTION OF PICORNAVIRUSES

2.1.5.1 POINT MUTATIONS

The evolution of RNA viruses is driven by two mechanisms, mutation and recombination. Mutations are based on the errors in the replication of the RNA catalyzed by the virus encoded RNA-dependent RNA polymerase (Holland et al. 1992). Since the polymerase lacks proof-reading activity, changes in the viral genome occur frequently. Rate of spontaneous mutations for enteroviruses have been estimated to be one mutation per genome per replication (Drake and Holland 1999). As a result, virus populations are genetically heterogeneous (Holland et al. 1982). These mixtures of genetic variants are called quasi-species (Holland et al. 1992). Most mutations generate synonymous codons which are unlikely to have effects on the genetic fitness of the virus. Selective environmental conditions may, however, shift the equilibrium of the virus population, providing an opportunity for any variant to compete with the dominant virus (Agol 1997). Enterovirus evolution is believed to occur primarily by genetic drift. Genetic drift develops, when a sample of quasi-species is amplified after passage through a genetic bottleneck (Holland and Domingo 1998). Most of the bottlenecks have been suggested to arise during replication within the human intestine (Hughes et al. 1988; Kinnunen et al. 1990). Neutral mode of evolution may also occur during person-to-person transmission of the virus. RNA viruses have been postulated to evolve conservatively, not adapting but merely changing (Sala and Wain-Hobson 2000).

2.1.5.2 RECOMBINATION

Another important means to extend the range of genetic variants for picornaviruses is recombination. During synthesis of the negative strand strand-switching occurs, frequently leading to replicative recombination (Agol 1997). Another suggested mechanism for recombination is nonreplicative, by strand-breaking and re-joining of RNA molecules by trans-esterification (Gmyl et al. 1999; Gmyl et al. 2003). Recombination of polioviruses was first discovered in the 1960s (Hirst 1962; Ledinko 1963). Experimental crosses of closely related strains have been shown to yield >1% recombinant progeny from a single growth cycle (Argos et al. 1984). Enteroviruses participate in intraserotypic and interserotypic homologous recombination (Santti et al. 1999; Andersson et al. 2002; Oprisan et al. 2002; Lindberg et al. 2003; Lukashev et al. 2003b). However, recombination has only been detected within species. Preferential

recombination breakpoints have been identified in the nonstructural part of the genome and at the 5'NCR-P1 junction (Santti et al. 1999; Andersson et al. 2002; Oprisan et al. 2002; Lindberg et al. 2003; Lukashev et al. 2003b). Recombination within the capsid coding region is rare, but has been reported for polioviruses among the OPV strains in the VP1 capsid protein coding region (Martin et al. 2002; Blomqvist et al. 2003). Recombination has been proposed to be a common trend in enterovirus genetics (Lukashev 2005). Recombination has not, however, been reported for rhinoviruses.

2.2 HUMAN RHINOVIRUSES

2.2.1 DESIGNATION OF DISTINCT SEROTYPES

Rhinovirus research commenced at the Common Cold Research Unit in England in the early 1950s (Tyrrell 2002). As serologically different strains were isolated, epidemiological data accumulated rapidly (Taylor-Robinson and Tyrrell 1962). The National Institute of Allergy and Infectious Diseases (NIAID) and the World Health Organization (WHO) initiated a *Rhinovirus Collaborative Programme* for the classification of rhinovirus strains. The antigenic relationships of a large collection of candidate rhinovirus strains were studied by a reciprocal neutralization test with virus strains and sera by a reference laboratory, the Children's Hospital in Columbus, Ohio. In addition, each laboratory submitting new strains was to test their candidate strains against all available sera. This procedure ensured two independent tests for new virus strains. HRVs were assigned a number and a prototype strain in three phases. The first phase assigned rhinoviruses from HRV1 to HRV55 (Kapikian et al. 1967). HRV1 had been shown to have two subtypes, HRV1A and HRV1B.

In the second phase, rhinoviruses were numbered from HRV56 to HRV89 (Kapikian et al. 1971). The third phase assigned rhinovirus serotypes HRV90 to HRV100 (Hamparian et al. 1987). After the *Rhinovirus Collaborative Program,* only one strain, Hanks, has been proposed as a new serotype (Gwaltney et al. 1978). However, in recent years, the serotypes of newly isolated HRV strains have not been systematically defined. Therefore, it is possible that new serotypes exist. As mentioned above, antigenic cross-reactions between serotypes occur, and some were noticed already during the numbering phases but were considered to be due to nonspecific inhibitors (Conant and Hamparian 1968).

2.2.2 GENERAL CHARACTERISTICS

Rhinoviruses share the basic properties of all viruses belonging to the family *Picornaviridae*. They are most closely related to enteroviruses (Fig. 2). Rhinoviruses and enteroviruses form two of the largest groups of Picornaviruses (Stanway et al. 2005). The classic difference observed between these virus genera has been their capacity to persist in an acidic environment. Enteroviruses have traditionally been thought to endure the acidic conditions of the stomach in order to be able to proceed to gut, where they replicate. Rhinoviruses, by contrast, infect primarily the tissue of the upper respiratory tract, and therefore do not need to withstand acidic conditions. The optimal pH for rhinoviruses ranges from 6.0 to 8.0. Below pH 6.0, rhinoviruses are readily inactivated. Infectivity of HRV14 has been shown to decrease at pH 5.0 in 20 minutes and totally disappear at pH 3.0 in 10 seconds. However, the inactivation in acidic conditions is dependent on the temperature. At pH 5.0, HRV14 loses 99% of its infectivity almost immediately at 24°C, but at 0°C remains infective for more than 5 minutes (Hughes et al. 1973). The molecular basis of the difference in acid sensitivity between entero- and rhinoviruses remains unknown. Irreversible conformational changes at both the surface of the virus and in the inner part of the capsid have been reported for HRV14 in acidic conditions (Giranda et al. 1992). However, adaptation to an acidic environment through mutation has been achieved by serial exposure of the viruses to pH 4.5 followed by passaging in HeLa cells (Skern et al. 1991).

The optimal temperature for rhinovirus isolation from clinical specimens is 33°C. However, their infectivity has been shown to be maintained at 24-37°C (Hendley et al. 1973) as well as at 6°C and 23°C (Reagan et al. 1981). They can survive on environmental surfaces for hours to days. In contrast to enteroviruses, most rhinoviruses are thermostable (Dimmock 1967). In laboratories, rhinovirus stocks, like the stocks of most viruses, are stored frozen. Their activity can be re-established after years of freezing (Couch 2001).

Rhinoviruses are resistant to ether, chloroform, fluorocarbon, and detergents due to the lack of a lipid envelope. Alcoholic disinfectants are not effective against rhinoviruses. (Couch 2001). Organic acids have been shown to have virucidal effects when added to hand cleansers (Turner et al. 2004). A mixture of peroxygen compounds and organic acids is generally used for virus inactivation in laboratories.

2.2.3 ANTIGENIC STRUCTURE AND NEUTRALIZATION MECHANISMS

The locations of neutralizing antigenic epitopes have been determined for HRV2 and HRV14 by analyzing neutralization-resistant mutants (Table 3). The epitopes were found in hypervariable regions of the capsid proteins located at the highest points of the virus surface. HRV14 was shown to have four different epitopes involved in antibody-mediated neutralization (Sherry and Rueckert 1985; Sherry et al. 1986), while three sites were involved in neutralization of HRV2 (Skern et al. 1987; Appleyard et al. 1990). Site A in HRV2 closely resembles the NImIA site of HRV14. Moreover, the VP2 component of site B of HRV2 has a partial analog in the NImII site of HRV14 (Skern et al. 1987; Appleyard et al. 1990; Verdaguer et al. 2000). Antibody-driven neutralization of rhinovirus infectivity may occur through several mechanisms, as reviewed by Smith 2001. These mechanisms are aggregation of viruses, stabilization of the viruses and subsequent prevention of uncoating, induction of conformational changes in the capsid, and steric blocking of cellular attachment, the last of which is considered the main mechanism.

Table 3. Amino acid residues defining the antigenic sites in Human rhinovirus (HRV) -2 and -14.*

	VP1	**VP2**	**VP3**
HRV2			
Site A	85,86,92		
Site B	260,262,264,265,272,274	159,161,163,164	59, 64
Site C		214,236,238	
HRV14			
NImIA	91,95		
NImIB	83,85,138,139		
NImII	210	156,158,159,161,162	
NImIII	287		72,75,78,203

*Adapted from Verdaguer et al. (2000)

2.2.4 RHINOVIRUS INFECTION

2.2.4.1 NATURAL COURSE AND CLINICAL PICTURE

Transmission of rhinovirus infection occurs through three routes: aerosols, fomites, and personal contact (Jennings and Dick 1987). Infection is initiated as the virus enters the nose or the eye, where it passes down the lacrimal duct. Rhinoviruses infect cells of the respiratory tract. They replicate mainly in the ciliary epithelial cells of the nasal mucosa and, to a lesser extent, the oral cavity and throat (Cate et al. 1964). Rhinoviruses have been suggested to infect cells of lower respiratory tract less often those of the upper respiratory tract. However, bronchial epithelial cells have also been shown to be susceptible to HRV (Papadopoulos et al. 1999; Hayden 2004). The amount of HRV needed to infect a human is very small, it is as little as one tissue culture infectious $dose_{50}$ ($TCID_{50}$).

Rhinoviruses are believed not to infect the intestine, attempts to infect the intestinal tract of adult volunteers have failed (Cate et al. 1967). Rhinoviremia is considered rare, although occasionally observed in fatal cases of infants (Urquhart and Stott 1970; Las Heras and Swanson 1983) and recently in several cases of asthma exacerbations in children (Xatzipsalti et al. 2005).

Rhinovirus infection mainly manifests as acute upper respiratory infection, generally known as the common cold. HRVs are responsible for most episodes of the common cold in all age groups (Arruda et al. 1997; Mäkelä et al. 1998). The common cold caused by HRV is clinically similar to that caused by other virus groups. The incubation period is 1-4 days, and the symptoms usually peak 3-7 days after the onset of infection (Couch 2001). Typical symptoms include sore throat, sneezing, nasal obstruction, and nasal discharge. Other symptoms can be hoarseness, cough, headache, malaise, and fever (Couch 2001). The duration of the illness is generally 7-14 days, but it can last longer. Viral shedding in nasal secretions can continue for up to three weeks, with the amount of virus in nasal secretions being small in the early days of infection (Gwaltney 2002a). Rhinoviral RNA can be detected in nasal secretions weeks after illness (Jartti et al. 2004). Symptoms of rhinovirus infection are believed to be partly caused by the induction of pro-inflammatory cytokines such as kinins, leukotrienes, histamines, interleukin-1, interleukin-6, interleukin-8, tumor necrosis factor, and RANTES (Naclerio et al. 1988; Noah et al. 1995; Schroth et al. 1999). Rhinovirus infection elicits serotype-specific immunity. Neutralizing antibodies have emerged in serum 14-17 days after inoculation, and peak titers were reached at 4-5 weeks following an experimental HRV15 infection (Douglas 1970). The titers were proposed to persist for at least 1-3 years.

Rhinovirus infection can range from a mild, self-limiting respiratory nuisance to a more severe outcome. Common complications of infection include acute otitis media (Arola et al. 1988; Pitkäranta et al. 1998; Vesa et al. 2001; Nokso-Koivisto et al. 2004), acute community-acquired sinusitis (Pitkäranta et al. 1997; Puhakka et al. 1998; Pitkäranta et al. 2001), and exacerbations of multiple types of pre-existing chronic respiratory diseases, such as asthma (Johnston et al. 1995; Gern 2002), chronic obstructive pulmonary disease (Greenberg 2002) and cystic fibrosis (Smyth 1995). Especially in children, rhinoviruses are often associated with such lower respiratory infections as bronchiolitis and pneumonia (Papadopoulos et al. 2000; Hayden 2004). Furthermore, rhinovirus infections can lead to severe or even fatal pneumonia in immunocompromised patients (Ghosh et al. 1999).

While rhinoviruses replicate in the upper respiratory pathways, enteroviruses replicate in the gut. Enteroviruses cause a wide range of diseases (Melnick 1996), with both individual and temporal variation. Most infections are mild or subclinical, proceeding over the course of several weeks. Rare cases, however, result in severe illness or even death. The clinical features of enteroviral diseases include poliomyelitis and other neurological disorders like meningitis, myocarditis, conjunctivitis, uveitis, skin and mucosal eruptions, diarrhea, generalized infection in newborns, and the common cold. Enteroviruses usually cause acute illnesses, but they have also been associated with such chronic diseases as dilated cardiomyopathy and type 1 diabetes (Hyöty et al. 1995; Dahlquist et al. 2004; Ylipaasto et al. 2004).

2.2.4.2 OCCURRENCE

Rhinoviruses infect humans of all ages, with children being the most susceptible (Cohen et al. 1997). Almost all children are reported to have experienced at least one rhinovirus infection by the age of two years (Blomqvist et al. 2002a). In the elderly, rhinovirus infections manifest as prolonged disease and lower tract symptoms (Hayden 2004). Exposure to cold and “chilling” did not appear to have a role in the etiology of the common cold in experimental infections (Douglas 1968). By contrast, significant psychosocial stress is likely to increase the occurrence and severity of the clinical symptoms of HRV infection (Cohen et al. 1997; Takkouche et al. 2001). Rhinoviruses cause infections year-round. The peak prevalence periods are, however, early fall and late spring months in the northern hemisphere (Cohen et al. 1997; Nokso-Koivisto et al. 2004). Enterovirus infections are most prominent in late summer and early fall (Melnick 1996).

2.2.4.3. TREATMENT

There is no specific treatment for rhinovirus infections. Several different approaches have been applied in the effort to find a treatment. These can be divided into symptomatic management aimed at improving the functional status of the patient, pharmacological treatment aimed at reducing the risk of complications, and antiviral agents aimed at reducing the concentration of virus, thus decreasing the likelihood of spread of the infection.

Symptomatic and pharmacological approaches include vitamin C (Schwartz et al. 1973), and Echinaceae extracts (Turner et al. 2000a; Turner et al. 2005). Anticholinergic compounds are directed against rhinorrhea produced by activation of the parasymphathetic nervous system (Gaffey et al. 1987a; Gaffey et al. 1988a). Antihistamines have been used to investigate the effect of blocking of the H_1 receptor against HRV colds (Gaffey et al. 1987b; Gaffey et al. 1988b; Gwaltney et al. 1996). Anti-inflammatory drugs with analgesic and antipyretic properties have also been tested (Hsia et al. 1989; Sperber et al. 1992), as have glucocorticoid steroids (Farr et al. 1990; Gustafson et al. 1996). In addition, several combinations of drugs from different groups have been evaluated. None of the above-mentioned compounds has proven a drug of choice for rhinovirus infections. Although some symptomatic efficacy has been shown, the common cold could not be prevented or cured with these drugs.

Antiviral drug research has produced new candidate rhinovirus drugs. Interferon-alpha (Hayden and Gwaltney 1984; Douglas et al. 1986), interferon-beta (Sperber et al. 1989) and zinc salts (Geist et al. 1987; Turner and Cetnarowski 2000b; Turner 2001) were the first antivirals tested. The most promising group at the moment is the capsid stabilizers, including pleconaril (Kaiser et al. 2000; Hayden et al. 2003a). However, the oral formulation had drug interactions due to the induction of CYP3A activity, leading to a reduction in the effectiveness of oral estrogen-based contraceptives, and thus, regulatory approval was not granted. Pleconaril is currently available on a compassionate use basis (Hayden 2004). Soluble ICAM-1 receptor molecules have also been tested, and tremacamra (Turner et al. 1999) reduced the severity of rhinoviral colds. The third group of potential antiviral drugs against rhinovirus infections are 3C protease inhibitors, including ruprintrivir (Hayden et al. 2003b). Antiviral treatment alone may cure the colds, but combining antivirals with selected therapeutic agents that block inflammatory pathways appears promising (Gwaltney 2002b).

2.2.5 LABORATORY DIAGNOSIS

2.2.5.1 VIRUS ISOLATION AND IDENTIFICATION OF RHINOVIRUS SEROTYPES

Traditionally, rhinoviruses have been detected in laboratories by isolation in cell culture (Couch 1992). Cell lines suitable for rhinovirus replication are derived from human or other primate tissue. This tissue specificity is affected by the presence of receptors on cell surfaces (Colonno et al. 1986). Recommendations for rhinovirus isolation are based on experience from early isolation attempts. Optimal culture conditions include a medium pH of 7.0-7.2, an incubation temperature of 33°C, and slow rotation of cultures (Couch 1992). Replication of the viruses is detected by appearance of cytopathic effects (CPEs). The isolation process followed by a subculture, including acid-sensitivity testing, is tedious and laborious, and is not generally attempted.

Identification of the serotype of isolated rhinovirus strains can be achieved by applying a neutralization test using a hyperimmune antisera. The test can be performed using a microneutralization assay with intersecting antiserum pools. The standard for serological identity of an unknown rhinovirus strain is neutralization of virus concentrations ranging from 10 to 300 $TCID_{50}$ by 20 units of antibody. The neutralization is carried out at 33°C for two hours. Completion of the assay takes 4-6 days. (Gwaltney et al. 1966; Couch 1992). The large number of serotypes and the laborious nature of the assay have hampered its use in rhinovirus diagnosics.

2.2.5.2 RT-PCR METHODS

Reverse transcriptase polymerase chain reaction (RT-PCR) has been widely used in rhinovirus detection since its introduction in the late 1980s (Gama et al. 1988; Gama et al. 1989; Hyypiä et al. 1989; Torgersen et al. 1989). Several RT-PCR applications have been described. Most of the rhinovirus RT-PCR assays take advantage of short, highly conserved stretches of nucleotides in the 5'NCR as the binding sites for oligonucleotide primers.

These assays have proven to be highly sensitive, but they do not differentiate rhinoviruses from enteroviruses. An extra step, such as analysis of restriction fragment length polymorphism (Torgersen et al. 1989), hybridization with rhinovirus-specific probes (Hyypiä et al. 1989; Johnston et al. 1993; Halonen et al.

1995; Blomqvist et al. 1999; Lönnrot et al. 1999; Andreoletti et al. 2000; Jenison et al. 2001), sequencing of PCR amplicons (Mori and Clewley 1994; Deffernez et al. 2004), semi-nested PCR (Ireland et al. 1993), or nested PCR with rhinovirus-specific primers (Andeweg et al. 1999; Steininger et al. 2001), is needed for differentiation between rhinoviruses and enteroviruses. Amplicon size can be used for differentiation when the RT-PCR is performed from the 5'NCR to the capsid coding region VP4 or VP2 (Olive et al. 1990). The binding site in VP2 is, however, variable leading to mismatching and reduced sensitivity (Santti et al. 1997; Hyypiä et al. 1998). The most recent application for rapid rhinovirus detection has been real-time PCR (Dagher et al. 2004; Kares et al. 2004).

2.3 MOLECULAR EPIDEMIOLOGY

2.3.1 DEFINITION AND GOALS

The glossary of molecular medicine defines molecular epidemiology as "the application of molecular biology to the answering of epidemiological questions" (Cambridge Healthtech Institute 2005). Another definition for molecular epidemiology is "epidemiology that focuses on measurements of molecular features" (National Cancer Institute 2005). Molecular epidemiology involves the integration of molecular biomarkers into population-based studies. The International Molecular Epidemiology Task Force (1998) has defined molecular epidemiology as "a science that focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the etiology, distribution, and prevention of disease within families and across populations". Molecular epidemiology has been used in the examination of patterns of changes in DNA to implicate particulate carcinogens. The use of molecular markers to predict which individuals are at highest risk for a disease has also served as an example of molecular epidemiology (Cambridge Healthtech Institute 2005). The term molecular epidemiology has frequently been applied in microbiological studies.

In microbiology and virology, molecular epidemiology means connecting certain genomic features of the pathogens to the distribution of related strains. The virological glossary defines molecular epidemiology as "the use of nucleotide sequence information to study the diversity and distribution of virus populations" (Microbiology @ Leicester 2004). Molecular epidemiological use of sequence information comes close to phylogenetics, which aims to estimate relationships and divergence of related sequences and to understand their past evolution.

2.3.2 METHODS AND LIMITATIONS

Molecular methods, such as genome amplification, restriction fragment length polymorphism, sequencing, and comparison of genetic information, have been widely used to elucidate the epidemiology of several viruses. Computerized analysis of the nucleotide and amino acid sequences can be used to infer phylogenetic relationships. The first step in the reconstruction of molecular phylogenetic relationships is generation of multiple sequence alignment of the selected sequences. The next step is mathematical modeling to describe the evolution of the sequences. Finally, a statistical method is applied to generate branching order and branch lengths best describing the sequence phylogenies. There are approximately 200 program packages using several different algorithms based on various evolutionary assumptions. Only some of the existing methods are discussed here.

The alignment of sequences can be performed with several programs. A commonly used global alignment program is ClustalX (Thompson et al. 1997), which uses a progressive method of clustering. The program creates a guide tree using the Neighbor-joining method (NJ) (Saitou and Nei 1987). Possible problems in alignment may be due to sequences of variable lengths (Palacios et al. 2002b). Another alignment program is the PileUp component of the GCG (Genetics Computer Group Inc., USA) program, which implements a simplified version of the Feng and Doolittle (1996) algorithm. This alignment tool can deal with terminal gaps. However, it assumes a molecular clock where mutations occur at the same rate in all sequences. This assumption may hold in a situation of very closely related sequences, but it poses limitations on the general use of the method.

The mathematical models for describing evolution can be divided into character-based and distance-based methods. Widely used character-based methods are maximum likelihood (ML) and maximum parsimony. Probabilistic likelihood techniques are based on the likelihood of a certain tree explaining the sequence data set. ML is statistically well founded, and a model of evolution is included in the analysis with ML. It is considered one of the most powerful methods of phylogenetic reconstruction. The major disadvantage of this method is that it is computationally intense. It can also be fooled by a high level of homoplasy, i.e. similarity between not homologous sequences. Quartet puzzling is a method of quartet-based maximum likelihood (Strimmer 1996). Maximum parsimony infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given data set. The main drawback of this method is that is underestimates the actual number of changes. Moreover, it does not use all sequence information (only informative sites), does not correct for multiple mutations (no model of evolution), does not provide information on branch lengths, and is sensitive to codon bias.

The distance-based methods calculate the distances between each pair of sequences and find a phylogenetic tree that reflects these distances. Distance methods in general use have been the Unweighted Pair Group Arithmetic Mean (UPGMA) (Sokal 1958) and currently the most popular NJ (Saitou and Nei 1987). The UPGMA is based on clustering of the sequences by similarity. The principle of NJ is to find pairs of neighbors that minimize the total branch length at each stage of clustering of neighbors, starting with a star-like tree. Terminal gaps in the sequences may pose a problem in the distance matrix estimation, but no signs of bias have also been reported (Kuhner and Felsenstein 1994). The main difference between these methods is the assumption of a molecular clock in the UPGMA. Distance methods are computationally very fast and suitable for large data sets. Some loss of evolutionary information data may occur when considering pairwise distances (Steel et al. 1988; Whelan et al. 2001). This is not, however, believed to compromise the accuracy of phylogeny estimation (Felsenstein 2004). The NJ method sometimes estimates negative branch lengths that are difficult to interpret (Kuhner and Felsenstein 1994).

Several models of nucleotide or amino acid substitution can be used for phylogenetic inference. Among the widely used models are Jukes-Cantor, the Kimura two parameter (K2P), Felsenstein 1981, Hasekawa, Kishino, and Yano (HKY85), General reversible and LogDet (Felsenstein 2005). Jukes-Cantor is a basic model assuming equal base frequencies and all substitutions at equal rates. The K2P model assumes equal nucleotide frequencies allowing, however, different rates for transitions and transversions. Felsenstein 1981 allows arbitrary base frequencies but equal rates for transitions and transversions. HKY85 allows unequal base frequencies and also provides a control for the transition/tranversion (ts/tv) ratio. General reversible allows both arbitrary base frequencies and substitution rates. The LogDet distance measure was developed to deal with sequences that differ significantly in nucleotide composition (Barry 1987). Besides these models, several others exist, and the choice of model depends on the sequence data set. In addition to the choice of the model, computer software includes various user-defined parameters, such as ts/tv ratio, heterogeneity in the rate of evolution, and proportion of invariant sites (Kuhner and Felsenstein 1994), that can affect the results of the analysis.

Further analysis to support the phylogenetic inference can be performed. Bootstrapping analysis is a resampling technique used to measure sampling error (Felsenstein 1985). It gives an idea about the reliability of branches and clusters. Quartet puzzling also allows investigation of the support of internal branching (Strimmer 1996). Likelihood mapping provides a means of visualizing phylogenetic content of the data set (Strimmer and von Haeseler 1997). It, like quartet puzzling, is

based on an analysis of the maximum likelihoods of the three fully resolved tree topologies computable for four sequences. It can be used to define whether the phylogeny of the data set is tree-, net-, or star-like.

2.3.3 MOLECULAR EPIDEMIOLOGY OF POLIOVIRUSES

Enterovirus research has long been focused on polioviruses (PVs). Molecular epidemiological methods have been widely used in the global Poliomyelitis Eradication Initiative (World Health Assembly 1988). Molecular epidemiology has provided a means to establish typical geographic distribution of distinct genetic lineages of PV (Rico-Hesse et al. 1987; Lipskaya et al. 1995; Mulders et al. 1995a). Furthermore, it has helped to identify genetic sources of outbreaks and sporadic cases of poliomyelitis (Pöyry et al. 1990; Kinnunen et al. 1991; Kew 1995; Mulders et al. 1995b; Mulders et al. 1997; Kew et al. 2002). The genotype demarcation for PV was determined as a 15% divergence in the 150-nt VP1/2A junction region of the genome (Rico-Hesse et al. 1987). This region was the traditional target for studies in PV, until the entire VP1 was shown to be the most reliable target for molecular epidemiological studies (reviewed in Kew et al. 2005).

2.3.4 MOLECULAR EPIDEMIOLOGY OF NON-POLIO ENTEROVIRUSES

Recently, genetic relationships of other emerging enteroviruses have been under extensive investigation. Molecular epidemiology of several HEV-B viruses, such as CV-A9 (Santti et al. 2000), CV-B4 (Mulders et al. 2000), CV-B5 (Kopecka et al. 1995), E-11 (Lukashev et al. 2002; Oberste et al. 2003a; Chevaliez et al. 2004), E-13 (Avellon et al. 2003; Mullins et al. 2004), and E-19 (Lukashev et al. 2003b), has been studied. The molecular epidemiology of other species of HEV, including CV-A24 (Dussart et al. 2005) and EV-71 (Brown et al. 1999), has also been examined. As a result of the studies, trends in the enterovirus genetics have been revealed. Enterovirus species are claimed to exist as a worldwide pool of genetic material that is constantly evolving (Lukashev et al. 2003a).

2.3.4.1 MOLECULAR TYPING OF ENTEROVIRUSES

The capsid protein coding region is the most variable in the picornavirus genome (Palmenberg 1989). The sequence of the capsid protein VP1 has been shown to

correlate well with the serotype of enteroviruses (Oberste et al. 1999b; Oberste et al. 1999c). Different approaches have been used to genetically define the “serotype” of an unknown enterovirus using either the entire VP1-coding gene (Oberste et al. 2000; Oberste et al. 2003b) or part of it (Caro et al. 2001; Norder et al. 2001; Palacios et al. 2002b; Thoelen et al. 2003). Criteria for sequence homology have been defined (Oberste et al. 2000). VP1 nucleotide sequence identity of more than 75% to a certain reference strain in GenBank indicates that the unknown sample is of homologous serotype, provided that the second-highest identity is less than 70%. Furthermore, the VP1 sequences of the same serotype have been demonstrated to form a homologous cluster in a phylogenetic tree (Oberste et al. 1999c; Caro et al. 2001; Norder et al. 2001). Since molecular typing has become widely used for the characterization of unknown enterovirus strains, new types have been assigned based on the sequence of the VP1 capsid protein coding gene. Recently, however, evidence has emerged that enteroviruses within a species may evolve as a genetic continuum, which obscures the 75% percentage nucleotide identity demarcation assigned for serotypes (Brown et al. 2005).

3 AIMS OF THE STUDY

GENERAL CONTEXT

Molecular tools (i.e. partial genome sequencing) have been widely used to elucidate the epidemiology of many important viruses such as polioviruses. Monitoring the progress of the global Poliomyelitis Eradication Initiative (Kew 1995; Mulders et al. 1997; Kew et al. 2005) has revealed interesting features of the genetic diversity of polioviruses and has brought along increasing interest towards non-polio enteroviruses. A series of studies on the molecular epidemiology of non-polio enteroviruses was started at the Enterovirus laboratory of the National Public Health Institute, Helsinki, Finland, to determine whether the evolutionary pattern of other HEVs including CV-B4 (Mulders et al. 2000) and CV-B5 (M. Mulders, pers. comm.), was similar to polioviruses. During the last decade E-30 has frequently been isolated in cases of aseptic meningitis in Europe. It was the increased epidemic activity of E-30 that prompted us to study its molecular epidemiology.

Another "activity" relevant to the current study was the efforts of the Finnish Otitis Media Cohort Study (FinOM Cohort) to characterize the role of specific pathogens in acute otitis media (AOM). The FinOM Cohort Study, carried out in Tampere, Finland, in 1994-1997, revealed that HRVs were frequently found in AOM cases in young children (Vesa et al. 2001). We therefore broadened our scope to include HRVs. At that time, little knowledge existed about the genetic relationships of HRVs and no molecular tools were yet available for HRVs. We had access to the FinOM Cohort field isolates of HRVs and developed molecular tools for their characterization. While processing them, it became clear that the genetic differences between different HRV strains were sufficiently large to enable "genetic typing" of HRVs. We therefore decided to perform systematic analysis of the partial genome sequences of all prototype strains of HRV.

Specific questions to which answers were sought:

1. What is the molecular epidemiological pattern of E-30 isolated in different parts of Europe?
2. What are the genetic relationships of recent field isolates of HRV?
3. Is it possible to identify the serotype of an HRV strain using partial genome sequencing in the capsid coding region?
4. Are the relationships of the HRV prototype strains different in distinct genomic regions, specifically in the capsid coding region and the RNA-dependent RNA polymerase coding region?

4 MATERIALS AND METHODS

The laboratory methods used are standard laboratory practices that are described in detail in the original articles (I-IV). Only a brief overview is given here.

4.1 VIRUS STRAINS

The E-30 field isolates had originally been isolated in different parts of Europe and other regions. The origins of the strains are provided in Study I. The prototype strain Bastianni was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Propagation of the strains and confirmation of serotypes are described in Study 1.

The HRV clinical samples (II-IV) isolated in the FinOM Cohort study, are described in detail in (Blomqvist et al. 1999) and are also listed in Table 4. The HRV prototype strains (III, IV) are listed in Table 5. The strains were either purchased from ATCC or received as a gift from the Haartman Institute, University of Helsinki, Finland; Janssen Pharmaceuticals, Beerse, Belgium; or the National Institute for Public Health and the Environment, Bilthoven, The Netherlands. HRV31 was also received from D. Blaas, University of Vienna, Austria. The HRV strain Hanks was provided courtesy of F. Hayden, University of Virginia, Charlottesville, VA, USA. The passaging of rhinoviruses is described in Studies II-IV.

Table 4. Human rhinovirus clinical isolates used in Studies II-IV.

Code	Sample type	Sample date	Code	Sample type	Sample date
6253sep94	NPA	140994	8022nov95	NPA	171195
6653oct94	NPA	031094	7137dec95	MEF	051295
7430oct94	NPA	281094	7425dec95	NPA	131295
7506mar95	NPA	240395	7052dec95	MEF	141295
9257apr95	NPA	050495	7113dec95	NPA	221295
5700may95	NPA	090595	6895jan96	NPA	270196
7931may95	NPA	230595	8570feb96	MEF	260296
5928may95	NPA	310595	8571feb96	NPA	260296
6562may95	NPA	310595	7922mar96	NPA	010396
6353jun95	NPA	080695	9316mar96	MEF	180396
7062jun95	NPA	160695	9317mar96	MEF	180396
9129jun95	NPA	210695	9318mar96	NPA	180396
8057jun95	NPA	290695	7181may96	NPA	020596
9702jul95	NPA	130795	6155may96	NPA	060596
6682jul95	NPA	170795	7389may96	NPA	220596
6322jul95	NPA	190795	7031may96	NPA	290596
8452jul95	NPA	210795	7746aug96	NPA	050896
7576aug95	NPA	220895	8643aug96	NPA	050896
8925sep95	NPA	050995	7910aug96	NPA	070896
6506sep95	NPA	080995	8169aug96	NPA	200896
7774sep95	NPA	120995	8317aug96	NPA	280896
6684sep95	NPA	200995	8507aug96	NPA	280896
6939sep95	NPA	240995	9863sep96	MEF	030996
6540sep95	MEF	250995	9864sep96	NPA	030996
9166sep95	NPA	270995	7850sep96	NPA	110996
6735oct95	NPA	041095	7790sep96	NPA	170996
7821oct95	MEF	171095	7851sep96	MEF	170996
7678nov95	NPA	011195	7852sep96	NPA	170996
7160nov95	NPA	041195	8358oct96	NPA	071096
7781nov95	NPA	151195	9056oct96	NPA	211096
8001nov95	NPA	151195			

Table 5. Human rhinovirus prototype strains used in Studies III and IV.

Serotype	Prototype strain	Origin*	Serotype	Prototype strain	Origin*
1A	Echo 28	RIVM	51	F01-4081	RIVM
1B	B632	RIVM	52	F01-3772	RIVM
2	HGP	HI	53	F01-3928	RIVM
3	FEB	RIVM	54	F01-3774	RIVM
4	16/60	RIVM	55	WIS 315E	RIVM
5	Norman	RIVM	56	CH82	RIVM
6	Thompson	RIVM	57	CH47	Janssen
7	68-CV 11	RIVM	58	21-CV 20	RIVM
8	MRH-CV 12	RIVM	59	611-CV 35	RIVM
9	211-CV 13	RIVM	60	2268-CV 37	RIVM
10	204 CV 14	RIVM	61	6669-CV 39	RIVM
11	1-CV 15	RIVM	62	1963M-CV 40	RIVM
12	181 CV 6	RIVM	63	6360-CV 41	RIVM
13	353	RIVM	64	6258-CV 44	RIVM
14	1059	RIVM	65	425-CV 47	RIVM
15	1734	RIVM	66	1983-CV 48	RIVM
16	11757	ATCC	67	1857-CV 51	RIVM
17	33342	RIVM	68	F02-2317-Wood	RIVM
18	5986-CV 17	RIVM	69	F02-2513-Mitchinson	RIVM
19	6072-CV 18	RIVM	70	F02-2547-Treganza	RIVM
20	15-CV 19	ATCC	71	SF365	RIVM
21	47-CV 21	RIVM	72	K2207	RIVM
22	127-CV 22	RIVM	73	107E	RIVM
23	5124-CV 24	RIVM	74	328A	RIVM
24	5146-CV 25	RIVM	75	328F	RIVM
25	5426-CV 12	RIVM	76	H00062	RIVM
26	5660-CV 27	RIVM	77	130-63	RIVM
27	5870 CV 28	RIVM	78	2030-65	RIVM
28	6101-CV 29	RIVM	79	101-1	RIVM
29	5582-CV 30	RIVM	80	277G	Janssen
30	106F	RIVM	81	483F2	Janssen
31	140F	RIVM, Vienna	82	3647	Janssen
32	363	RIVM	83	Baylor 7	Janssen
33	1200	RIVM	84	432D	Janssen
34	137-3	RIVM	85	50-525-CV-54	Janssen
35	164A	ATCC	86	121564-Johnson	Janssen
36	342H	RIVM	87	F02-3607-Corn	ATCC
37	151-1	RIVM	88	CVD-01-0165-Dambrauskas	Janssen
38	CH79	HI	89	41467-Gallo	Janssen
39	209	RIVM	90	K2305	Janssen
40	1794	RIVM	91	JM1	Janssen
41	56110	RIVM	92	SF-1662	Janssen
42	56822	RIVM	93	SF-1492	Janssen
43	58750	ATCC	94	SF-1803	Janssen
44	71560	Janssen	95	SF-998	ATCC
45	Baylor 1	RIVM	96	SF-1426	Janssen
46	Baylor 2	RIVM	97	SF-1372	Janssen
47	Baylor 3	RIVM	98	SF-4006	Janssen
48	1505	RIVM	99	604	Janssen
49	8213	RIVM	100	K6579	Janssen
50	A2 No.58	RIVM	unnumbered	Hanks	Charlottesville

***** RIVM, National Institute for Public Health and the Environment, Bilthoven, The Netherlands; HI, Haartman Institute, University of Helsinki, Finland; ATCC, American Type Culture Collection, Manassas, VA, USA; Janssen, Janssen Pharmaceuticals, Beerse, Belgium; Vienna, D. Blaas, University of Vienna, Austria; Charlottesville, F. Hayden, University of Virginia, Charlottesville, VA, USA.

4.2 RHINOVIRUS ISOLATION IN CELL CULTURE AND RNA ISOLATION

A rhinovirus-sensitive strain of the HeLa cell line provided by E. Arruda (University of Virginia, Charlottesville, VA, USA) was used for the isolation of rhinoviruses (II-IV). Rhinovirus isolation procedure for rolling tubes at 33°C was applied (Couch 1992). The RNA was isolated from 100 µl cell culture homogenate with a commercial RNA isolation kit (RNeasy Total RNA kit, Qiagen, GmbH, Hilden, Germany). RNA extracts were frozen and stored at -70°C until use.

4.3 RT-PCR AND DETECTION OF AMPLICONS

The procedure for RT-PCR has been described in Mulders et al. (2000). The cDNA synthesis was carried out for 1 h in a reaction volume of 10 µl, which contained 1 µl of heat-denatured RNA, 25 mM Tris–HCl (pH 8.3 at 25°C), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 1 mM dNTPs (Boehringer Mannheim), 2 U avian myeloblastosis virus reverse transcriptase (Finnzymes), 4 U RNase inhibitor (Promega), and 12.5 pmol of primer. A mixture of mineral oil (Sigma) and DynaWax (Finnzymes) in ratio of 7:1 was applied as overlay. Upon completion of the RT reaction, the sample was heated at 95°C for 5 min and then chilled to 4°C. For PCR, the reaction volume was adjusted to 50 µl by adding 4 µl of 10x PCR buffer (0.1 M Tris–HCl, pH 8.8 at 25°C, 0.5 M KCl, 1% Triton X-100), 0.75 µl of 50 mM $MgCl_2$, 12.5 pmol of both primers, 1 U recombinant *Thermus brockianus* DNA polymerase (DyNAzyme II; Finnzymes), and distilled water. Cycling conditions were as follows: 1 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 42°C, and 2 min 30 s at 72°C. The cDNA synthesis and PCR were performed as separate steps in Studies I-III. In Study IV, RT-PCR were carried out in a single-tube fashion (Oberste et al. 2000). The oligonucleotide primers used in all studies are provided in Table 6. The PCR products were visualized after electrophoresis in an ethidium bromide –stained 2% agarose gel.

Table 6. Oligonucleotide primers used in Studies I-IV.

Primer code	Location	Sequence 5' - 3'	Orientation	Reference	Study
71693	449-470*	CCT CCG GCC CCT GAA TGC GGC	F	Rotbart 1990	I
9895	534-560**	GGG ACC AAC TAC TTT GGG TGT CCG TGT	F	Study II	I,II, III
580	591-609*	GGC TGC TTA TGC TGA CAA T	F	Mulders et al., 2000	I
9565	1083-1058**	GCA TCI GGY ARY TTC CAC CAC CAN CC	R	Study II	I,II, III
72438	1211-1192*	GGC AAC TTC CAC CAC CAC CC	R	Mulders et al., 2000	I
81294	1211-1195*	GGC AAC TTC CAC CAC CA	R	Modified from Olive et al. 1990	I
8839	2388-2407*	TGC TTT GTG TCA GCA TGC AA	F	Mulders et al., 2000	I
82138	2626-2645*	TAC CAC ACC AGA TCA GAG TC	F	Study I	I
8840	2884-2906*	ACA CAC CAA ATA ATG TAC GTG CC	F	Mulders et al., 2000	I
81494	2935-2958*	AAC AGT TAC AGC TGG CAG ACA TC	F	Study I	I
8841	2993-2974*	GCA TTG CCC TCT GTC CAA AA	R	Mulders et al., 2000	I
82139	3120-3100*	CAA GTG TCC CAT GTT GTT CAA	R	Study I	I
73124	3437-3418*	TCC CAC ACG CAA TTT TGC CA	R	Mulders et al., 2000	I
3DF	6559-6578**	GGI GGI RTN CCM TCN GGN TG	F	Study IV	IV
3DR	7131-7154**	T_{21}ATA	R	Study IV	IV

*Location according to the complete genome sequence of EV-30 Bastianni (AF162711). ** Location according to the complete genome sequence of HRV1B (Hughes et al. 1988). I= inosine, N= A, T, C, G; Y= T, C; R= G, A; M=C, A. F= forward, R= reverse.

4.4 SEQUENCING

PCR products were purified prior to sequencing either directly using a PCR Purification kit (QIAquick, Qiagen) or excised from the gel and purified using the Gel Extraction kit (QIAquick, Qiagen). The purified products were stored at -20°C or directly used in cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Espoo, Finland). Automated sequencer ABI PRISM 377 was used for sequencing in Studies I-IV and ABI PRISM 310 in Study IV.

4.5 SEQUENCE ANALYSIS

Sequence data were analyzed with Sequencing Analysis (version 3.1; Applied Biosystems) and Sequence Navigator (version 1, Applied Biosystems). Multiple sequence alignments were performed with the PileUp (I) component of the GCG program (version 10, Genetics Computer Group, Inc., USA) and ClustalX version 1.64b (II, III) or 1.83 (IV) (Thompson et al. 1997). Alignments were generated with default parameters and adjusted manually. Distance matrices were estimated using DNAdist and PROTdist from PHYLIP [Phylogeny Inference Package, version 3.572c, (I-III); 3.6 (IV)] (Kuhner and Felsenstein 1994) using a ML method (I-III) and the K2P model (IV). The dendrograms were drawn with Neighbor (PHYLIP) using the UPGMA (I) and NJ (II-IV) options. The dendrograms were visualized with Njplot (Perriere 1996) or Treeview version 1.5.3 (Page 1996). Bootstrap analysis (Hillis 1993) was performed using Seqboot (PHYLIP) with 100 or 1000 replicates. Quartet puzzling (I) was performed with PUZZLE (version 4.0) (Strimmer 1996) with 1000 replicates. The likelihood mapping analysis (II) was performed with PUZZLE (version 4.0) (Strimmer and von Haeseler 1997) with 1000 random quartets and the HKY85 model of substitution (Hasegawa et al. 1985).

Similarity analysis and Bootscanning (Salminen et al. 1995) were used to identify recombinant strains in the complete genome alignments. They were performed with SimPlot software package version 2.5 or 3.5.1 (Lole et al. 1999). SimPlot calculates and plots the identity (%) of the query sequences to reference sequences in a sliding window. Nonrecombinant sequences show homology at a similar level throughout the genome, whereas for recombinants the highest homology sequence depends on the region. In bootscanning, a query sequence is aligned with reference sequences, phylogenetic trees are constructed, bootstrapping analysis is performed, and bootstrap values are plotted in segments. Nonrecombinant sequences show high

bootstrap values with one reference strain throughout the genome, while recombinants cluster with two or more parental strains. A sliding window of 200 nucleotides moving in steps of 20 nucleotides was used for both analyses. For bootscanning, the NJ method and K2P model of substitution were used, and the ts/tv ratio was estimated from the data set. Positions containing gaps were excluded from analyses.

5 RESULTS AND DISCUSSION

5.1 MOLECULAR EPIDEMIOLOGY OF ECHOVIRUS 30 CLINICAL ISOLATES (I)

E-30 was included in the series of studies on molecular epidemiology of HEV-B viruses because of its clinical significance. One of the neurologic complications of enteroviruses is aseptic meningitis. Echoviruses have been found to be responsible for 80-90% of all viral meningitis (Diedrich et al. 1995). During the 1990s E-30 was frequently isolated in cases of aseptic meningitis (Mori et al. 1995; Hovi et al. 1996; Maguire et al. 1999). E-30 strains isolated in distinct areas had been shown to vary genetically (Gjoen et al. 1995). To illustrate the molecular epidemiological pattern of European E-30 isolates, we sequenced three genomic regions of 129 E-30 field isolates. While we were processing these, the epidemic activity of E-30 continued to increase. Two other groups published a similar characterization focusing on different geographical areas and distinct genomic regions: in Scotland 5'NCR and VP4/VP2 were studied (Nairn and Clements 1999) and in USA the VP1 region (Oberste et al. 1999a). A study of 13 German strains on a partial VP1 region was also published (Kunkel and Schreier 2000). A specific diagnostic PCR for E-30 has subsequently been developed (Kilpatrick et al. 2001), and a report on E-30 isolations in USA has been published (Centers for Disease Control and Prevention 2003). A study summarizing 318 different E-30 strains worldwide was published in 2002 by Palacios et al. (2002a). Recently, persistent circulation (Chambon et al. 2001; Bernit et al. 2004) and sporadic outbreaks of E-30 have been described in various parts of the world (Manzara et al. 2002; Wang et al. 2002; Ozkaya et al. 2003; Vestergaard et al. 2004). A RFLP-based identification tool has also been developed to monitor cases of viral meningitis with regard to E-30 (Charrel et al. 2004).

The genetic regions in our study of E-30 included a 420-nt stretch in the VP4/VP2 coding region, the entire VP1 (876 nt), and a 150-nt VP1/2A region. All 130 E-30 strains, including the prototype strain Bastianni, were sequenced in the VP4/VP2 region, and based on the observed clustering, a subset of strains was sequenced in other regions. E-30 was found to differ from other analyzed enteroviruses in that the genetic variation of E-30 strains was markedly lower in all studied regions. However, the amino acid variation of E-30 in VP1 exceeded that found for CV-B4, although the nucleotide variation was lower.

Several different co-circulating genetic lineages have been found for PVs (Rico-Hesse et al. 1987; Mulders et al. 1995a), CV-A9 (Santti et al. 2000), CV-B4

(Mulders et al. 2000), CV-B5 (M. Mulders, pers. comm.), E-11 (Oberste et al. 2003a), and CV-A24 (Dussart et al. 2005), while a single genotype of E-30 has prevailed in Europe for more than two decades (Study I). The prevailing strains differ significantly from the prototype Bastianni, as also shown by others (Oberste et al. 1999a; Kunkel and Schreier 2000). Furthermore, the previous genetic lineages were found to be replaced by the dominant lineage. This epidemiological pattern of E-30, where prevailing lineages displace less established ones, as described in Study I, resembles that of influenzaviruses A and B, a view shared by others (Palacios et al. 2002a). In influenzaviruses the prevailing lineages are selected on the basis of immune escape (Scholtissek 1996). In addition, the observation of relatively large amino acid variation in VP1 among the studied E-30 strains could indicate antigenic differences. This study did not include characterization of possible antigenic alterations. Later antigenic analyses of E-30 field strains have not revealed a pattern connected to genetic clustering, although differences exist (C. Savolainen-Kopra, unpublished data). Differences in ability to cause functional impairment and cell death in cultured human pancreatic β-cells have subsequently been demonstrated among E-30 strains of distinct molecular lineages (Roivainen et al. 2002). Certain strains of E-30, not clustering together, though, in Study I, were clearly more destructive than the E-30 prototype strain Bastianni. Recently, the mode of epidemiology found for E-30, successive replacement of prevailing clusters, has been suggested to describe the current situation with several enteroviruses, being, however, less clear-cut for some serotypes (Lukashev 2005).

Besides European strains, our study included one strain from Japan, two strains from Russia, two strains from Byelorussia, and two strains from Israel. All of these clustered among the prevailing genotype. However, worldwide, evidence has emerged of more than one co-circulating genotype (Oberste et al. 1999a; Bailly et al. 2002; Palacios et al. 2002a; Wang et al. 2002). Figure 3 illustrates the prevailing genotype also found widely outside Europe and the separate genotypes formed by recent isolates from the Philippines and Colombia. The existence of a predominant genetic type is said to correlate with the temporal dynamics of E-30 isolation (Oberste et al. 1999a). Recent reports have shown strains of E-30 frequently involved in recombination (Lukashev et al. 2003a; Oberste et al. 2004a). The nonstructural part of the E-30 Bastianni genome has spread through recombination to many other serotypes isolated in the late 1990s (Lukashev et al. 2003a). While this is not likely to be a special characteristic of E-30 Bastianni, it does support the suggestion of Lindberg and coworkers (2003) that P2-P3 regions can be universally used among different HEV-B viruses. Several virtually disappeared genotypes of E-30 may provide important gene transfer to newer strains of other serotypes of HEV.

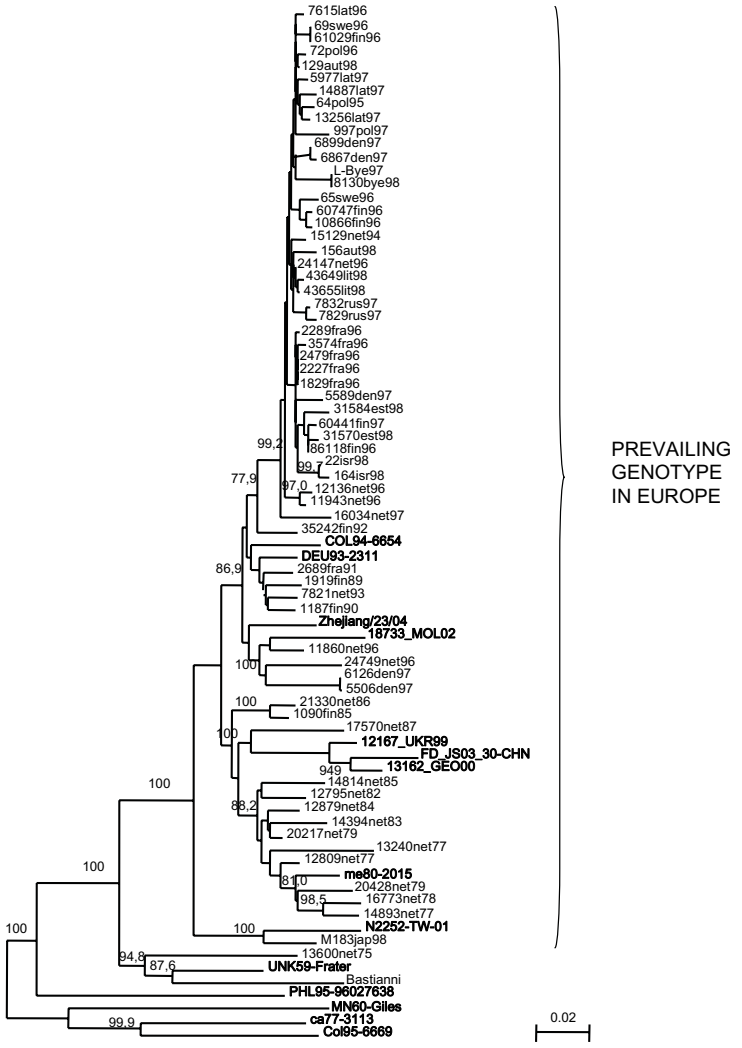

Figure 3. Neighbor-joining dendrogram of the E-30 VP1 sequences (876 nt) (Study I) added with GenBank sequences published in other studies (in boldface). Numbers indicate Bootstrap values (1000 replicates); only values >70% are shown.

5.2 GENETIC RELATIONSHIPS OF CLINICAL ISOLATES OF HUMAN RHINOVIRUSES FROM SUCCESSIVE EPIDEMIC SEASONS (II)

An opportunity opened up for us to study genetic diversity and molecular evolution of HRV field strains and compare these with the closely related HEV, as in the FinOM Cohort study, which focused on the role of different risk factors in AOM, 833 rhinovirus-positive recently collected specimens were found (Blomqvist et al. 2002a; Nokso-Koivisto et al. 2004). From these, a random selection of 61 samples was included in the analysis of genetic diversity. The sequencing was initiated from the genomic region coding for capsid proteins VP4/VP2. In enteroviruses, this region is known to be flanked by relatively conserved stretches, suitable for designing primers for strains of unknown sequence. Previous studies (Duechler et al. 1987; Horsnell et al. 1995) have shown that HRVs can be divided into two major genetic clades. The larger of the clades is related to HRV1B and comprises the majority of the strains analyzed thus far. The smaller clade is related to HRV14. The genetic and evolutionary differences between these clades are marked, as can be seen in Figure 4. Since these studies, very little new information on the genetic relationships of currently circulating strains has arisen.

The reliability of phylogenetic information and the suitability of tree-like dendrograms for the alignment of rhinoviral sequences were tested with Likelihood mapping analysis (Strimmer and von Haeseler 1997). The results for both genetic clades indicate a well-resolved phylogeny and do not support a star-like evolution. Thus, we concluded that phylogenetic reconstruction in the form of a tree-like dendrogram was suitable for rhinoviruses. However, 10% of the test tree topologies in genetic group I of the Likelihood mapping analysis represent phylogenetic noise, possibly caused by a star-like evolution. In genetic group II, this value was 1.5%. This can also be seen in the longer terminal branches of the dendrogram of group I as compared with group II (Study II, Fig. 2). A possible intepretation of this might be that the strains in genetic group I have a longer time interval from the first diversions to this point. The tree of group II has branched more frequently during the same time interval.

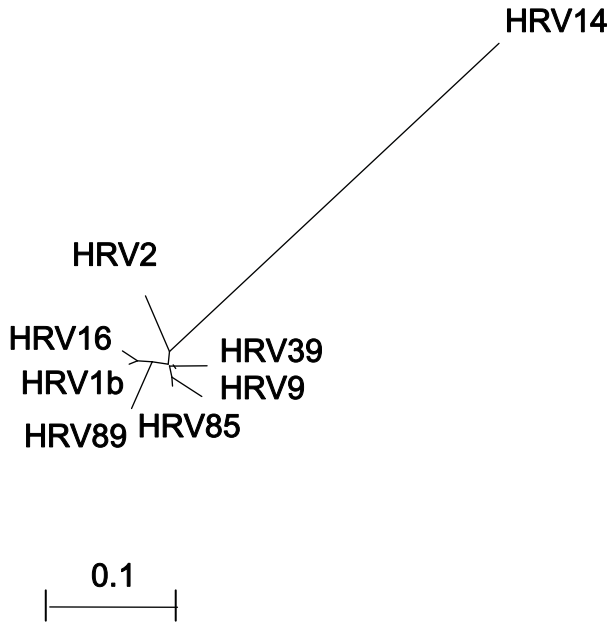

Figure 4. Phylogenetic relationships of the completely sequenced HRV strains in the VP4/VP2 capsid coding region. Neighbor-joining dendrogram of 140 amino acids.

Based on the clustering in the VP4/VP2 region, the sequences of the 61 analyzed rhinovirus strains were divided into the two formerly known clades. The HRV1B-related group comprised 24 of the clinical isolates, while 37 isolates clustered in the HRV14-related group. The sequences formed temporal clusters covering one epidemic season. However, certain clusters had members isolated in successive seasons. In addition, co-circulation of separate genetic lineages was found. Furthermore, reappearance of a cluster after disappearing for a given season was seen. This might, however, be due to the limited number of strains studied. Several separate genetic lineages, possibly representing serotype-like clusters, could be observed among the studied rhinovirus field isolates. The range of evolutionary distances was very large, from identical sequences to more than 30% divergence. With an approximate evolutionary distance of 20% or more, certain field strains could be linked to a prototype strain, even though the analysis included only five completely sequenced prototype strains. An example of this is a pair formed by 5700may95 and HRV16.

5.3 ALL BUT ONE HUMAN RHINOVIRUS PROTOTYPE STRAINS CLUSTER IN THE TWO KNOWN CLADES IN THE CAPSID REGION (III)

As the genetic relationships of HRV field strains appeared complex (Study II), we performed a systematic analysis of all designated prototype strains of HRV in the same genetic region. A total of 97 HRV prototype strains were sequenced in the VP4/VP2 coding region. The previously sequenced five prototype strains were included in the analysis. The strains included two subtypes of HRV1 and an unnumbered suggested new serotype Hanks. Seventy-six strains clustered in the HRV1B-related group, designated HRV-A. Twenty-five strains clustered in the HRV14-related group, designated HRV-B. HRV87 did not cluster with either of the major clades, but was found to be close to EV-70. HRV87 has subsequently been further characterized and reclassified as a member of HEV-D (Blomqvist et al. 2002b). The division of HRV prototype strains into two clusters is illustrated as a dendrogram in Figure 5. The evolutionary variation maxima within the two groups were 41% and 34% at the nucleotide level and 28% and 20% at the amino acid level, respectively. These differences are roughly the same as those found for enteroviruses in the capsid coding region. In the publication of Study III, the HRV31 sequence included in the dendrogram was erroneous; HRV31 had been contaminated with HRV32 (Vlasak et al. 2003). The correct HRV31 was obtained from Vienna and the sequence revised in GenBank in June 2003 (AF343583). The revised sequence is included in Figure 5. Subclusters or distinct lineages of HRV can be seen, but the branching is not well supported by Bootstrapping. Furthermore, the model of nucleotide substitution used has some effect on tree topology, albeit the members of the sublineages stay coherent regardless of the method of evolutionary reconstruction.

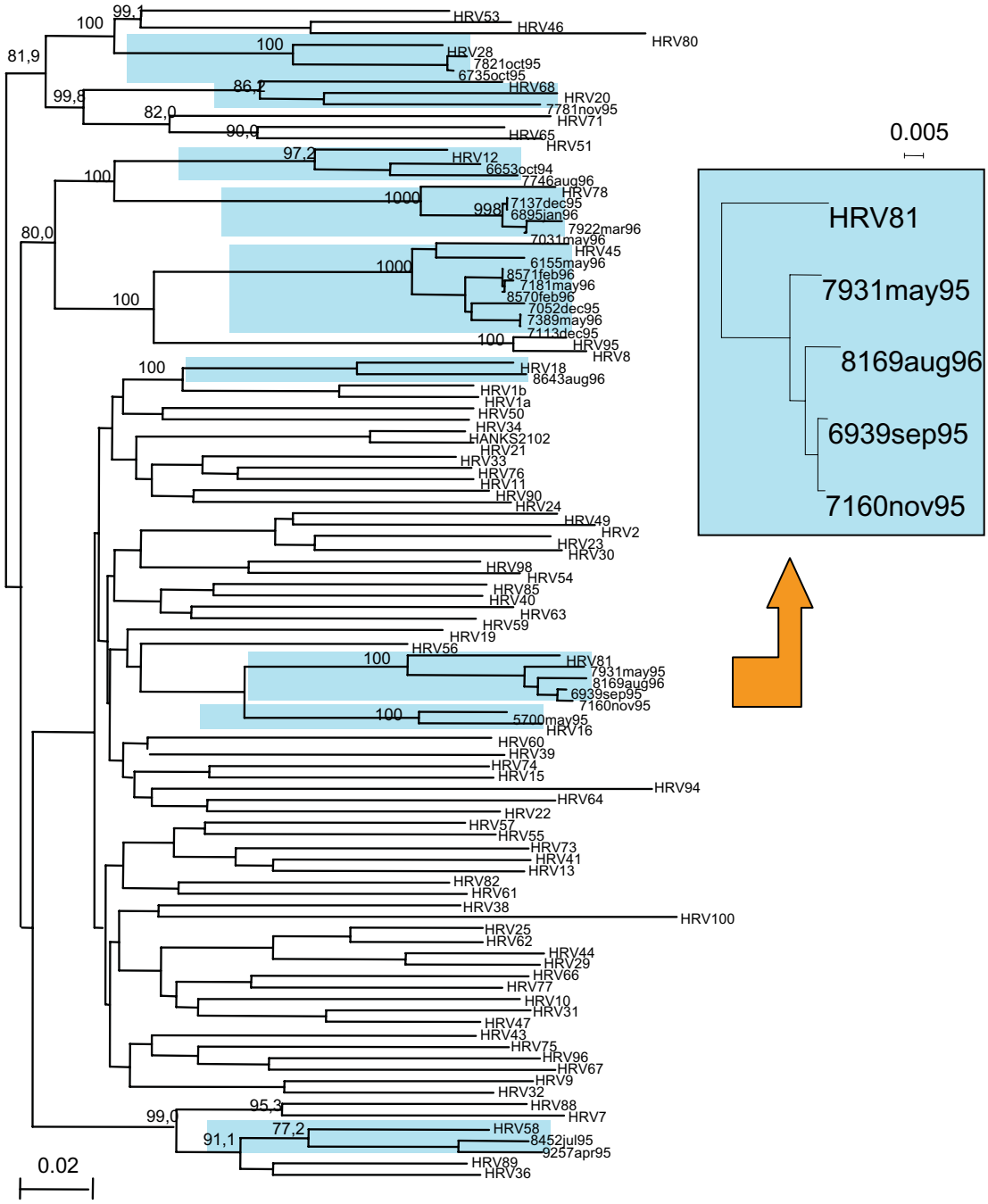

Figure 5. a) Neighbor-joining dendrogram of HRV-A strains in the VP4/VP2 capsid coding region (420 nt). Numbers indicate Bootstrap values (1000 replicates); only values >70% are shown. The blue bar indicates an approximate demarcation of 20%. One cluster of field isolates from different epidemic seasons and the closest prototype strain highlighted.

Figure 5. b) Neighbor-joining dendrogram of HRV-B strains in the VP4/VP2 capsid coding region (420 nt). Numbers indicate Bootstrap values (1000 replicates); only values >70% are shown. The blue bar indicates an approximate demarcation of 20%.

Judging from the phylogenetic tree in the VP4/VP2 region, the field isolates might represent as many as 19 different serotypes (Fig. 5a and b). However, as noted before for enteroviruses (Oberste et al. 1998; Oberste et al. 1999b; Oberste et al. 1999c; Mulders et al. 2000), the capsid coding region outside VP1 may not always be conclusive in defining the serotype. Furthermore, determining the closest prototype strain to field strains is difficult because five pairs of prototype strains (HRV25/62, HRV29/44, HRV8/95, HRV1A/1B, HRV21/Hanks) have an evolutionary distance of less than 10%. The VP1 sequences of all HRV prototype strains have since been published (Ledford et al. 2004; Laine et al. 2005). The overall pattern with the major clades and clusters was identical to the one seen in VP4/VP2. The same prototype strains were found to be close to each other also in the VP1 coding part of the genome. We therefore suggest that in the genetic typing of HRV the term "the closest related prototype strain" be used instead of aiming at a definite serotype. HRV do not show a coherent distance pattern throughout all serotypes in the capsid region, but exist as a genetic continuum within the major clades HRV-A and HRV-B. This is similar to the variation of viruses within HEV-C (Brown et al. 2005). Other groups have subsequently published sequence data on rhinoviruses isolated from respiratory samples in GenBank (Arden et al. 2004; Diedrich et al. 2004). These data suggest results similar to those of Studies II and III; sequences of field isolates cluster close to a prototype strain, but the divergence from the second closest prototype strain is not much larger.

Strong genetic support for antigenic cross-reactivity emerges, as all pairs reported to have reciprocal cross-reactions (Cooney et al. 1973; Cooney et al. 1982) cluster together in VP4/VP2. In the figures of Study III, HRV12 and HRV78 did not cluster as close as in the dendrogram based on the K2P model (Fig. 5). Several pairs of one-way cross-reactions (Cooney and Kenny 1970; Calhoun et al. 1974; Cooney et al. 1982) also cluster close to each other, but some exist in different branches; however, always in the same major clade. Antigenic cross-reactions have not been studied for HRV8 and HRV95. They have only four divergent nucleotides and no amino acid changes in the 420-nt stretch in VP4/VP2. VP4/VP2 region, however, contains no amino acids of the known antigenic sites of rhinoviruses (Sherry and Rueckert 1985; Sherry et al. 1986; Skern et al. 1987; Verdaguer et al. 2000). Genetic closeness of HRV8 and HRV95 has subsequently been seen in VP1 (Ledford et al. 2004; Laine et al. 2005), and they have been suggested to be classified in a single serotype similar to HRV21 and HRVHanks (Ledford et al. 2004).

This study was the first systematic investigation of the capsid sequences of HRV prototype strains. It has been used as a basis for classification of rhinoviruses by the Picornavirus Study Group of the International Committee for the Taxonomy of Viruses (Stanway et al. 2005). Furthermore, the finding of the outlier nature of HRV87 originally made here and further studied by Blomqvist et al. (2002) and others (Ishiko et al. 2002; Ledford et al. 2004; Oberste et al. 2004b) has led to the classification of HRV87 as a member of HEV-D. These studies have prompted further discussions on the separation of entero- and rhinoviruses based on acid sensitivity. The titer of enterovirus 68, another strain of the same serotype as HRV87, was reduced by incubation at pH 3.0 (Blomqvist et al. 2002b; Oberste et al. 2004b), although enteroviruses are generally considered acid-stable. Furthermore, all serotypes and strains of rhinoviruses may not be acid-labile (S. Blomqvist, pers. comm.). These issues have led to discussions and an official proposal by the Picornavirus Study Group that the enterovirus and rhinovirus genera should be combined to form a single genus (T. Hovi, pers. comm.).

5.4 GENETIC CLUSTERING OF RHINOVIRUSES IN THE NONSTRUCTURAL PART OF THE GENOME SUPPORTS DIFFERENCES IN THE PHYLOGENETIC HISTORY OF STRAINS (IV)

To enlarge the general picture of genetic relationships of HRV strains, we sequenced 48 HRV prototype strains and 12 field strains in the partial RNA-dependent RNA polymerase (3D) coding part of the genome. The length of the analyzed part of the 3D was 468 nt. A similar distribution to the major clades was seen as in the capsid part of the genome. All field strains clustered close to the same prototype strains as in the capsid region. Some differences in tree topology could be seen in HRV-A as compared with the VP4/VP2 tree. However, our analysis included only a subset of HRV serotypes, and it is possible that the sequences of the remaining prototype strains would affect the alignment.

The location of one cluster of strains in the HRV-A dendrogram differed strikingly from the clustering seen in the VP4/VP2 region. This group comprised HRV8, HRV95, HRV45, and 6155may96. In 3D they were clearly separated from the rest of the HRV-A strains and designated as HRV-A'. Figure 6 displays the genetic relationships of 43 HRV-A prototype and field strains, including HRV-A'. HRV-A' also formed a subcluster in the VP4/VP2, but nevertheless clustered within HRV-A. In VP1 and 2A, this group has been shown to form a cluster clearly distinct from HRV-A

(Laine et al. 2005). HRV-A' may represent a group with a phylogenetic history that is different from that of other HRV and HEV. Different genomic regions of HRV-A' may have evolved at different rates. The variation in the 3D part of the genome in HRV was generally larger than that seen in the correspondent part of HEV. Furthermore, HRV-B clustered closer to HEV than to HRV-A, unlike in the VP4/VP2 region. This is in accordance with the findings of Stanway et al. (1984) regarding HRV14.

5.5 EVIDENCE FOR RECOMBINATION WITHIN HUMAN RHINOVIRUS GENOMES

The incongruence in the tree topology between the capsid region and the nonstructural region led us to suspect possible recombination events within HRV-A. This discrepancy is illustrated in a combined analysis of two distant segments of HRV prototype strains, the complete genome sequence of which has been published (Fig. 7). Furthermore, the long terminal branches, seen in the dendrograms of HRV-A have been considered as possible evidence of recombination (Schierup and Hein 2000). Other reasons for long terminal branches might be heterogeneity in the rate of sequence evolution or bias in the ts/tv ratio. However, both of these have been observed to have an effect smaller than recombination (Schierup and Hein 2000). The default ts/tv ratio of 2 was used in the analyses. In Study II, the ratios were determined from the data sets. The ratios for other genomic regions have also been determined from the data sets (data not shown), and the default value of 2 was found to be applicable. In addition to long terminal branches, recombination can lead to more star-shaped genealogy (Schierup and Hein 2000). Some evidence of this was seen in Study II in the Likelihood mapping analysis of genetic group I (HRV-A), although the results still indicated a tree-like phylogeny. As variation among different HRV strains is large and different regions in the HRV genome might evolve at different rates, recombination may be difficult to prove based on partial genome sequences. As stated for HEV-B (Oberste et al. 2004a), evidence of recombination may be obscured by accumulation of nucleotide sustitutions.

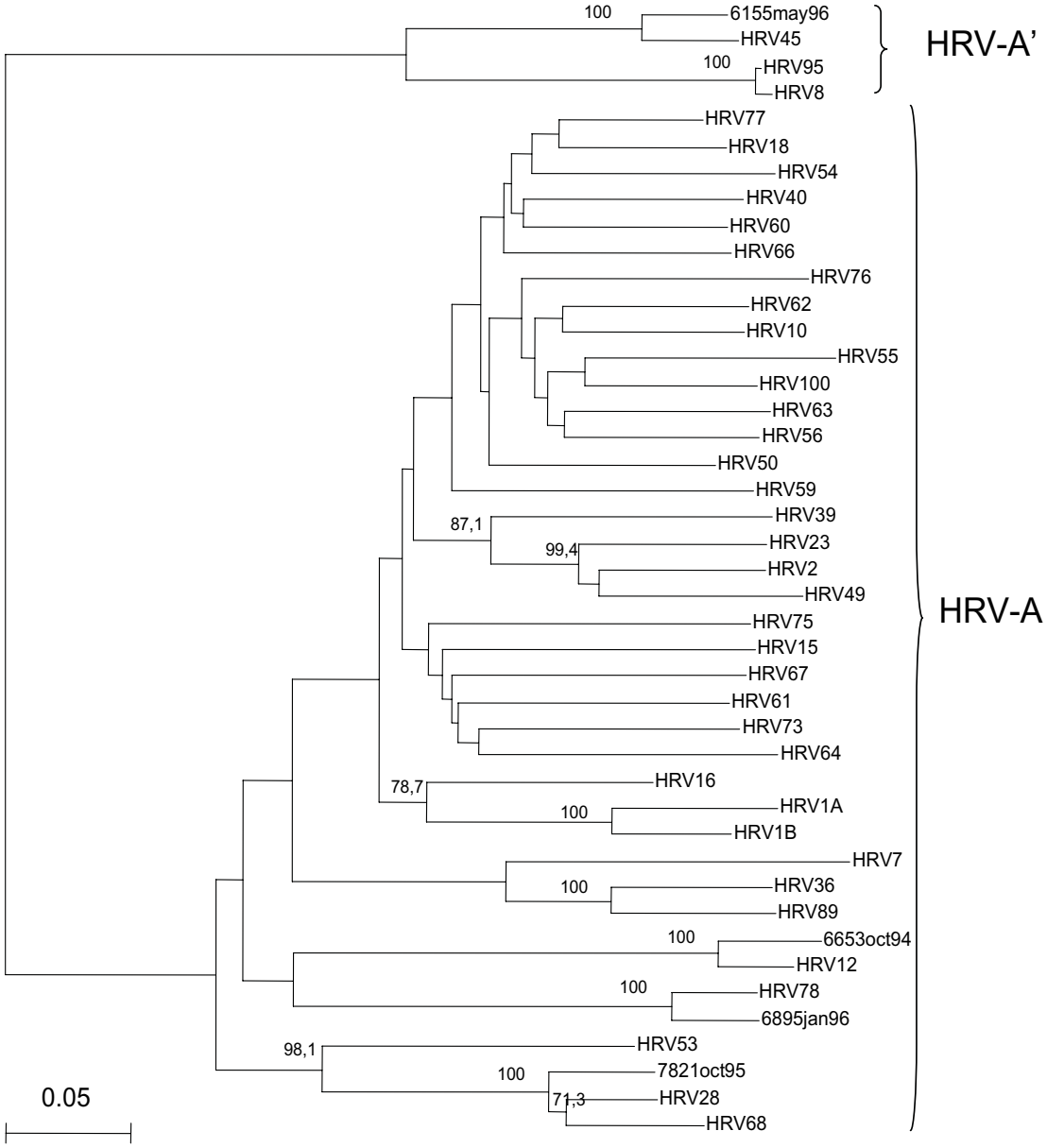

Figure 6. Neighbor-joining dendrogram depicting phylogenetic relationships of HRV-A in the RNA-dependent RNA polymerase (3D) coding region (468 nt). Numbers indicate Bootstrap values (1000 replicates); only values >70% are shown.

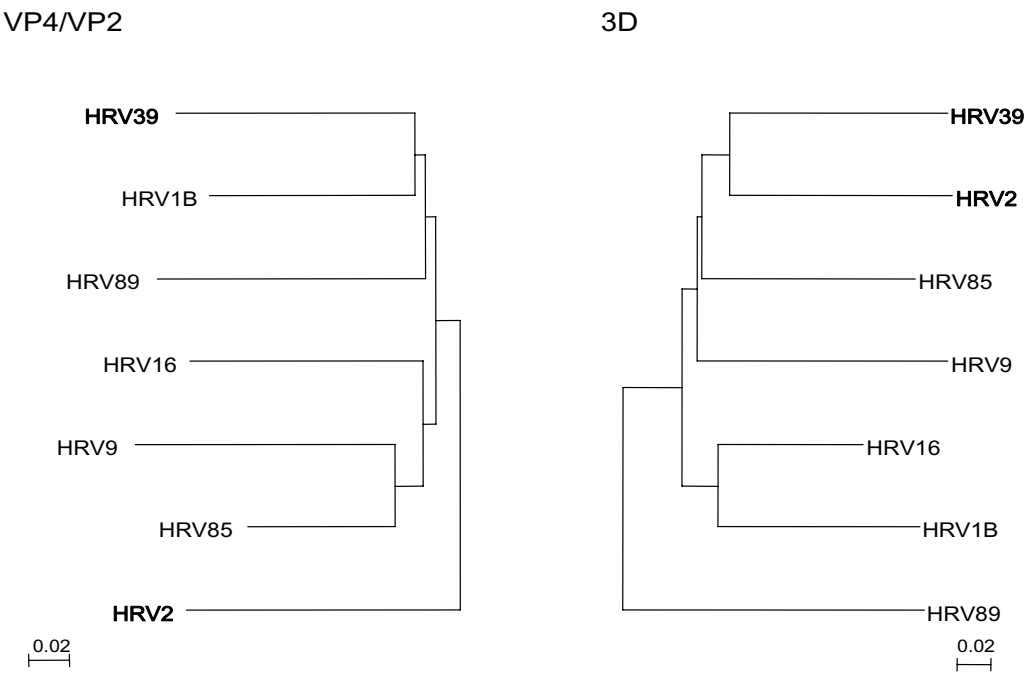

Figure 7. Combined phylogenetic analysis of HRV prototype strains, the complete genome sequence of which has been published in the VP4/VP2 capsid coding region (left side) and in the RNA-dependent RNA polymerase (3D) coding region (right side).

To elucidate the recombination hypothesis, all available HRV-A complete genome sequences (HRV1B D00239, HRV2 X02316, HRV9 (Leckie 1987), HRV16 L24917, HRV39 AY751783, HRV85 (Stanway 1989), and HRV89 NC_001617) were plotted against each other in the SimPlot and Bootscan analyses. The results of these analyses for HRV2 are displayed in Figure 8. Most of the studied HRV-A genomes showed background similarity to each other, rarely exceeding 80%, except in the 5'NCR (data not shown). However, HRV2 and HRV39 were more closely related in the nonstructural part of the genome in Similarity plotting. High support for recombination between HRV2 and HRV39 was seen in Bootscanning analysis. The recombination breakpoint was localized in 2A (around nt 3220). However, due to the large genetic variation among all HRV-A strains, the exact location could not be pinpointed. Moreover, it is impossible to determine whether HRV39 is the real parental strain or is it perhaps another strain of HRV, the complete genome of which is unavailable. A recombination event may have happened early in the evolution of

HRV2 and HRV39, but several subsequent point mutations have blurred the relationship to the parental strains. Interestingly, HRV2 belongs to the minor receptor group of HRV, whereas HRV39 is a member of the major receptor group. This could mean that the two viruses have simultaneously infected the same human cell with different receptors. The isolation years for prototype strains HRV2 and HRV39 are 1957 and 1962, respectively (Taylor-Robinson and Tyrrell 1962; Mufson et al. 1965), which does not rule out the possibility that they were circulating at the same time. However, as theoretically shown in the recombination evidence for HEV-B, two strains being related by recombination does not necessarily mean these serotypes were simultaneously present in the same host cell; the observed recombination may be due to serial recombination events (Oberste et al. 2004a). The same can be true for HRV-A

Recombination has been shown to be frequent among different HEVs (Santti et al. 1999; Andersson et al. 2002; Oprisan et al. 2002; Lindberg et al. 2003; Lukashev et al. 2003a; Lukashev et al. 2004). Similar to HEV, several HRV strains circulate simultaneously (Study II), and due to the large number of serotypes, it is likely that double infections occur. Why recombination was not observed among the field strains in our study may be because of the small number of strains sequenced in the 3D region. Another reason why recombination is not seen more often in HRV might be the short duration of infection. Recombination has been considered to increase fitness of PV. The HRV recombinant progeny may be less fit to enrich in the virus population. However, only a small subset of HRV has thus far been completely sequenced. Information on complete genome sequences of HRV is needed to shed light on recombination events. Furthermore, it has been said for enteroviruses that knowing the serotype of an enterovirus isolate only means that one-third of its genome is more than 70% similar to the prototype strain isolated in the 1950s (Lukashev 2005). This has been considered the reason why it is so difficult to correlate an enterovirus serotype with the clinical manifestation. Recombination or gene transfer in the nonstructural part of the genome may also have important consequences in the pathogenicity of HRVs.

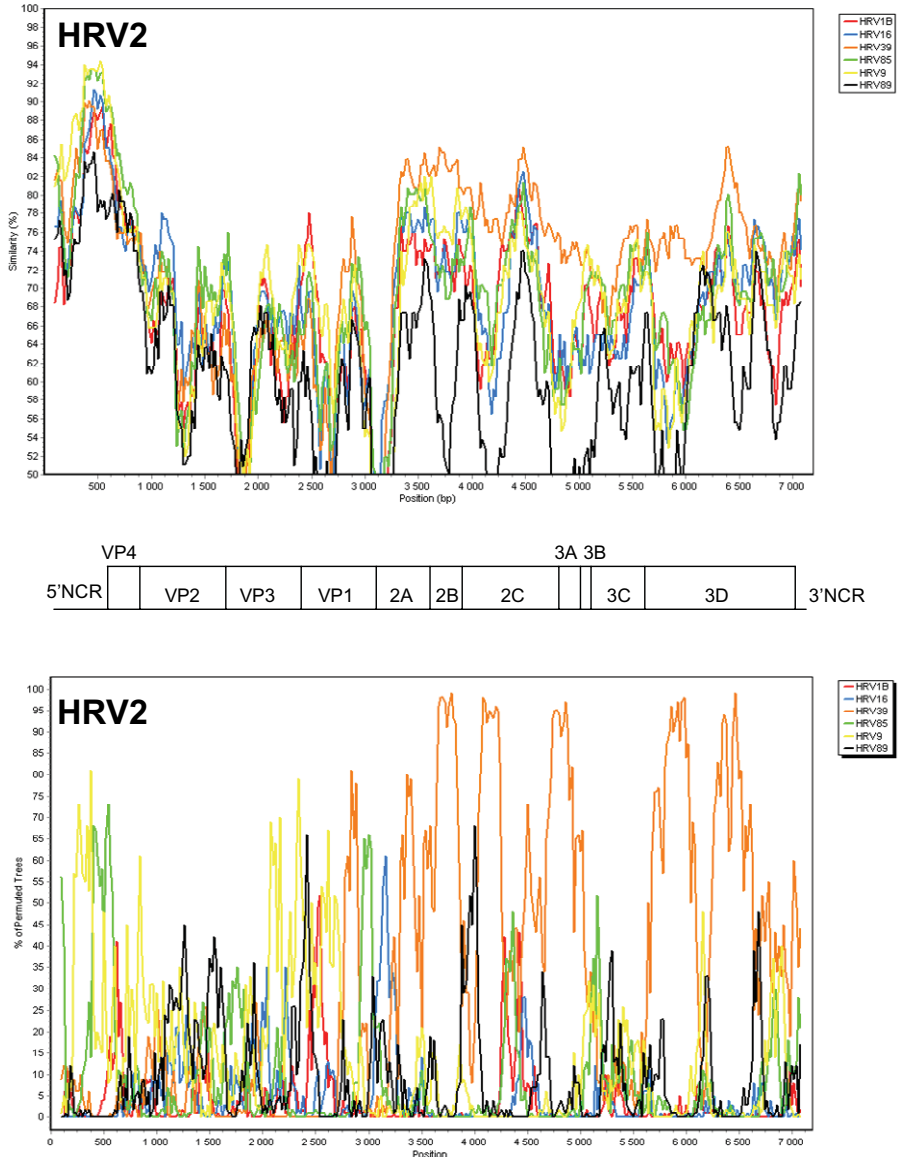

Figure 8. Complete genome sequence of Human rhinovirus (HRV) -2 plotted against other available complete genome HRV sequences. Upper panel: Similarity plotting analysis; lower panel: Bootscan analysis with Neighbor-joining method. A sliding window of 200 nt and steps of 20 nt were used for both analyses.

6 CONCLUSIONS

As part of a series of studies elucidating molecular epidemiological relationships of HEV-B viruses, a prevalent enterovirus, echovirus 30 (E-30), was analyzed by partial genome sequencing of a total of 129 European field isolates. Compared with many other enteroviruses, including poliovirus, an atypical pattern was seen, with the prevailing genetic lineage replacing the previous lineages.

A large collection of HRV-containing respiratory samples was available from another study, the FinOM Cohort Study, which aimed at characterizing the role of specific pathogens in AOM. To determine molecular epidemiological relationships of the close relatives of HEV, 61 HRV field strains were examined by sequencing of the VP4/VP2 capsid protein coding region. Currently circulating HRV field isolates were shown to cluster in the two established major genetic clades in the VP4/VP2 genomic region. More than 60% of the strains clustered in the HRV14-related clade. Several co-circulating strains were observed, possibly representing different serotypes of HRV.

To investigate the possibility of genetic typing of HRV, we analyzed all known 102 HRV prototype strains in the genetic region coding for VP4/VP2. The first systematic genetic characterization of all designated HRV prototype strains was performed. All prototype strains, except HRV87, were shown to cluster in the two major clades, designated *Human rhinovirus A* (HRV-A) and *Human rhinovirus B* (HRV-B). The clades comprised 76 and 25 prototype strains, respectively. The field isolates could be linked to a prototype strain, enabling genetic typing of HRVs with the criterion “the closest prototype strain”.

A total of 50 HRV prototype and 12 field strains were genetically analyzed in the RNA-dependent RNA polymerase coding region (3D). The same major clades were seen as in the capsid region. The variation of HRV in the 3D region was larger than that observed in the VP4/VP2 capsid coding region. This observation is in contrast to HEV, for which the variation in the capsid region exceeds that of the 3D region. Furthermore, the variation of HRV was found to exceed that of HEV in the 3D region. The general phylogenetic tree topology differed from that for the capsid coding region among HRV-A strains, suggesting possible recombination events.

Evidence for recombination was obtained with Similarity and Bootscanning analyses of previously published complete genome sequences. Putative recombination was detected among the genomes of HRV2 and HRV39.

In this work, we have used phylogenetic methods to elucidate the genetic relationships of a group of viruses with large variation that hampers the development of medicines and vaccines. We have shown the genetic distribution of both the prototype strains of HRV and a collection of recently isolated field strains. Our results together with subsequently published VP1 data for HRV provide a taxonomic classification of all designated HRV prototype strains.

7 ACKNOWLEDGMENTS

This work was performed at the Enterovirus Laboratory of the Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland.

I thank Professors Jussi Huttunen and Pekka Puska, the former and present Head of the Institute, respectively, for placing excellent facilities at my disposal.

The Steering Group of the FinOM studies is thanked for the opportunity to work with the material derived from these studies.

My warmest gratitude is due to my supervisor, Professor Tapani Hovi, Head of the Department. His vast knowledge of virology and ability to see the possibilities behind the jungle of information available never cease to amaze me. Discussions with him have invariably infused me with hope and optimism.

Professor Timo Hyypiä and Docent Alexander Plyusnin are acknowledged for their expert review of my thesis and invaluable comments. Carol Ann Pelli is kindly thanked for reviewing the language of the manuscript. Petri Ylipaasto is thanked for helping with the cover graphic.

Special thanks go to Mick Mulders, who introduced me to enteroviruses and sequencing. His enthusiasm and sense of humor lightened also the dark days after he left our lab. My scientific career was off to a good start thanks to Mick.

I thank Docent Merja Roivainen, Head of the Enterovirus Laboratory, for giving me time to concentrate solely on my writing. I learned much during this process.

Without Soile Blomqvist, my work simply would not have been finished. Soile was an enormous help and support at the beginning of my first maternity leave, when part of the work was still unfinished. Furthermore, Soile's "feet on the ground" attitude was a perfect balance for my own rollercoaster mind.

Pia Laine, another rollercoaster mind, is warmly thanked for sharing the lab, office, and fears during sequencing of endless rhinovirus prototype strains. Our friendship goes beyond viruses.

I feel lucky to have received practical guidance in the lab from Päivi Hirttiö. Päivi has been a great help with technical and everyday issues.

Mika Salminen is thanked for patient help with phylogenetic issues. All members of the HIV laboratory in KTL are thanked for collaboration.

Mirja Stenvik is acknowledged for friendship at work and outside of work. Johanna Nokso-Koivisto was a welcome visitor in our lab and is thanked for great companionship. Antero Airaksinen and Taina Härkönen were senior PhD students when I began this project. They are thanked for their inspiring example and many fun conversations. I am grateful to Eija Penttilä, Mervi Eskelinen, and Elisa Lamminsalo, all my office-mates and other people in the Enterovirus Laboratory for creating such a pleasant work atmosphere.

I owe a great deal to my parents, Sinikka and Pauli. Thank you for your love and continuous support as well as for your common sense, which helped me keep my scientific problems in perspective. Thanks also for taking care of Marcus and Christa whenever needed.

My late grandmother, Mummi, is warmly remembered for her unconditional love and unquestioning faith in me.

Now that I am already in tears, I thank you, Kimmo, for loving me more than I deserve. I am grateful for the household chores that you do to give me time for my work. Marcus and Christa, our beloved children, are thanked for showing me every day how exciting it is to learn and discover new things. You are all an enormous support and I love you dearly!

This work has been partly supported by grants from the Academy of Finland, the Commission of the European Communities, the Päivikki and Sakari Sohlberg Foundation, the Sigfrid Juselius Foundation, and Wyeth-Lederle Vaccines and Pediatrics.

Helsinki, 9.12.2005

Carita Savolainen-Kopra

8 REFERENCES

Abraham, G. and Colonno, R. J. (1984). "Many rhinovirus serotypes share the same cellular receptor." J Virol **51**(2): 340-5.

Agol, V. (1997). "Recombination and other genomic rearrangements in picornaviruses." Seminars in Virology **8**: 77-84.

Al-Nakib, W., Stanway, G., Forsyth, M., Hughes, P. J., Almond, J. W. and Tyrrell, D. A. (1986). "Detection of human rhinoviruses and their molecular relationship using cDNA probes." J Med Virol **20**(3): 289-96.

Andersson, P., Edman, K. and Lindberg, A. M. (2002). "Molecular analysis of the echovirus 18 prototype: evidence of interserotypic recombination with echovirus 9." Virus Res **85**(1): 71-83.

Andeweg, A. C., Bestebroer, T. M., Huybreghs, M., Kimman, T. G. and de Jong, J. C. (1999). "Improved detection of rhinoviruses in clinical samples by using a newly developed nested reverse transcription-PCR assay." J Clin Microbiol **37**(3): 524-30.

Andreoletti, L., Lesay, M., Deschildre, A., Lambert, V., Dewilde, A. and Wattre, P. (2000). "Differential detection of rhinoviruses and enteroviruses RNA sequences associated with classical immunofluorescence assay detection of respiratory virus antigens in nasopharyngeal swabs from infants with bronchiolitis." J Med Virol **61**(3): 341-6.

Andries, K., Dewindt, B., Snoeks, J., Wouters, L., Moereels, H., Lewi, P. J. and Janssen, P. A. (1990). "Two groups of rhinoviruses revealed by a panel of antiviral compounds present sequence divergence and differential pathogenicity." J Virol **64**(3): 1117-23.

Andries, K., Dewindt, B., Snoeks, J., Willebrords, R., Stokbroekx, R. and Lewi, P. J. (1991). "A comparative test of fifteen compounds against all known human rhinovirus serotypes as a basis for a more rational screening program." Antiviral Res **16**(3): 213-25.

Appleyard, G., Russell, S. M., Clarke, B. E., Speller, S. A., Trowbridge, M. and Vadolas, J. (1990). "Neutralization epitopes of human rhinovirus type 2." J Gen Virol **71 (Pt 6)**: 1275-82.

Arden, K. E., McErlean, P., Sloots, T. P. and Mackay, I. M. (2004). "Improved detection rates demonstrate rhinoviruses in significant numbers among pediatric patients with acute respiratory disease." unpublished.

Argos, P., Kamer, G., Nicklin, M. J. and Wimmer, E. (1984). "Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families." Nucleic Acids Res **12**(18): 7251-67.

Arola, M., Ziegler, T., Ruuskanen, O., Mertsola, J., Nanto-Salonen, K. and Halonen, P. (1988). "Rhinovirus in acute otitis media." J Pediatr **113**(4): 693-5.

Arruda, E., Pitkäranta, A., Witek, T. J., Jr., Doyle, C. A. and Hayden, F. G. (1997). "Frequency and natural history of rhinovirus infections in adults during autumn." J Clin Microbiol **35**(11): 2864-8.

Avellon, A., Casas, I., Trallero, G., Perez, C., Tenorio, A. and Palacios, G. (2003). "Molecular analysis of echovirus 13 isolates and aseptic meningitis, Spain." Emerg Infect Dis **9**(8): 934-41.

Bailly, J. L., Brosson, D., Archimbaud, C., Chambon, M., Henquell, C. and Peigue-Lafeuille, H. (2002). "Genetic diversity of echovirus 30 during a meningitis outbreak, demonstrated by direct molecular typing from cerebrospinal fluid." J Med Virol **68**(4): 558-67.

Barry, D., and Hartigan, J.A. (1987). "Statistical analysis of hominoid molecular evolution." Statistical Science **2**: 191-210.

Bernit, E., de Lamballerie, X., Zandotti, C., Berger, P., Veit, V., Schleinitz, N., de Micco, P., Harle, J. R. and Charrel, R. N. (2004). "Prospective investigation of a large outbreak of meningitis due to echovirus 30 during summer 2000 in Marseilles, France." Medicine (Baltimore) **83**(4): 245-53.

Blomqvist, S., Skyttä, A., Roivainen, M. and Hovi, T. (1999). "Rapid detection of human rhinoviruses in nasopharyngeal aspirates by a microwell reverse transcription-PCR-hybridization assay." J Clin Microbiol **37**(9): 2813-6.

Blomqvist, S., Roivainen, M., Puhakka, T., Kleemola, M. and Hovi, T. (2002a). "Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children." J Med Virol **66**(2): 263-8.

Blomqvist, S., Savolainen, C., Råman, L., Roivainen, M. and Hovi, T. (2002b). "Human rhinovirus 87 and enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features." J Clin Microbiol **40**(11): 4218-23.

Blomqvist, S., Bruu, A. L., Stenvik, M. and Hovi, T. (2003). "Characterization of a recombinant type 3/type 2 poliovirus isolated from a healthy vaccinee and containing a chimeric capsid protein VP1." J Gen Virol **84**(Pt 3): 573-80.

Brown, B. A., Oberste, M. S., Alexander, J. P., Jr., Kennett, M. L. and Pallansch, M. A. (1999). "Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998." J Virol **73**(12): 9969-75.

Brown, B., Maher, K., Naragni-Arani, P., Oberste, S., and and Pallansch, M. (2005). Variability of the capsid region of human enterovirus species C clinical isolates: serotypes as descrete genetic clusters or sampling of a genetic continuum within species. EUROPIC, European Study Group on the Molecular Biology of Picornaviruses Lunteren, The Netherlands.

Calhoun, A. M., Jordan, W. S., Jr. and Gwaltney, J. M., Jr. (1974). "Rhinovirus infections in an industrial population. V. Change in distribution of serotypes." Am J Epidemiol **99**(1): 58-64.

Cambridge Healthtech Institute. (2005). "Molecular Medicine Glossary." Retrieved 24.10.2005, from http://www.genomicglossaries.com/content/clinical_genomics_email.asp.

Caro, V., Guillot, S., Delpeyroux, F. and Crainic, R. (2001). "Molecular strategy for 'serotyping' of human enteroviruses." J Gen Virol **82**(Pt 1): 79-91.

Casasnovas, J. M. and Springer, T. A. (1994). "Pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released." J Virol **68**(9): 5882-9.

Cate, T. R., Couch, R. B. and Johnson, K. M. (1964). "Studies with Rhinoviruses in Volunteers: Production of Illness, Effect of Naturallly Acquired Antibody, and Demonstration of a Protective Effect Not Associated with Serum Antibody." J Clin Invest **43**: 56-67.

Cate, T. R., Douglas, R. G., Jr., Johnson, K. M., Couch, R. B. and Knight, V. (1967). "Studies on the inability of rhinovirus to survive and replicate in the intestinal tract of volunteers." Proc Soc Exp Biol Med **124**(4): 1290-5.

Centers for Disease Control and Prevention (2003). "Outbreaks of aseptic meningitis associated with echoviruses 9 and 30 and preliminary surveillance report on enterovirus activity - United States, 2003." MMWR: 761-783.

Chambon, M., Archimbaud, C., Bailly, J. L., Henquell, C., Regagnon, C., Charbonne, F. and Peigue-Lafeuille, H. (2001). "Circulation of enteroviruses and persistence of meningitis cases in the winter of 1999-2000." J Med Virol **65**(2): 340-7.

Charrel, R. N., Bernit, E., Zandotti, C. and de Lamballerie, X. (2004). "An approach based on RFLP assay to investigate outbreaks of enteroviral meningitis." J Clin Virol **29**(1): 54-8.

Chevaliez, S., Szendroi, A., Caro, V., Balanant, J., Guillot, S., Berencsi, G. and Delpeyroux, F. (2004). "Molecular comparison of echovirus 11 strains circulating in Europe during an epidemic of multisystem hemorrhagic disease of infants indicates that evolution generally occurs by recombination." Virology **325**(1): 56-70.

Cohen, S., Doyle, W. J., Skoner, D. P., Rabin, B. S. and Gwaltney, J. M., Jr. (1997). "Social ties and susceptibility to the common cold." JAMA **277**(24): 1940-4.

Colonno, R. J., Callahan, P. L. and Long, W. J. (1986). "Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses." J Virol **57**(1): 7-12.

Committee on the ECHO Viruses (1955). Enteric cytopathogenic human orphan (ECHO) viruses. Science. **122:** 1187-1188.

Conant, R. M. and Hamparian, V. V. (1968). "Rhinoviruses: basis for a numbering system. II. Serologic characterization of prototype strains." J Immunol **100**(1): 114-9.

Cooney, M. K. and Kenny, G. E. (1970). "Reciprocal neutralizing cross-reaction between rhinovirus types 9 and 32." J Immunol **105**(2): 531-3.

Cooney, M. K., Kenny, G. E., Tam, R. and Fox, J. P. (1973). "Cross relationships among 37 rhinoviruses demonstrated by virus neutralization with potent monotypic rabbit antisera." Infect Immun **7**(3): 335-40.

Cooney, M. K., Fox, J. P. and Kenny, G. E. (1982). "Antigenic groupings of 90 rhinovirus serotypes." Infect Immun **37**(2): 642-7.

Couch, R. (1992). Rhinoviruses. Laboratory Diagnosis of Viral Infections. E. H. Lennette. New York, Marcel Dekker Inc.**:** 709-729.

Couch, R. B. (2001). Rhinoviruses. Fields Virology. 4th ed. D. M. Knipe, and Howley, P.M. Philadelphia, Lippincott Williams & Wilkins**:** 777-797.

Crump, C. E., Arruda, E. and Hayden, F. G. (1993). "In vitro inhibitory activity of soluble ICAM-1 for the numbered serotypes of human rhinovirus." Antiviral Chem Chemoth **4**: 323-327.

Dagher, H., Donninger, H., Hutchinson, P., Ghildyal, R. and Bardin, P. (2004). "Rhinovirus detection: comparison of real-time and conventional PCR." J Virol Methods **117**(2): 113-21.

Dahlquist, G. G., Forsberg, J., Hagenfeldt, L., Boman, J. and Juto, P. (2004). "Increased prevalence of enteroviral RNA in blood spots from newborn children who later developed type 1 diabetes: a population-based case-control study." Diabetes Care **27**(1): 285-6.

Deffernez, C., Wunderli, W., Thomas, Y., Yerly, S., Perrin, L. and Kaiser, L. (2004). "Amplicon sequencing and improved detection of human rhinovirus in respiratory samples." J Clin Microbiol **42**(7): 3212-8.

Diedrich, S., Driesel, G. and Schreier, E. (1995). "Sequence comparison of echovirus type 30 isolates to other enteroviruses in the 5'noncoding region." J Med Virol **46**(2): 148-52.

Diedrich, S., Scheweiger, B., and and Schreier, E. (2004). "Detection of human rhinoviruses from respiratory samples in Germany." unpublished.

Dimmock, N. J. (1967). "Differences between the thermal inactivation of picornaviruses at "high" and "low" temperatures." Virology **31**(2): 338-53.

Douglas, R. G., Jr., Cate, T. R. and Couch, R. B. (1966). "Growth and cytopathic effect of H type rhinoviruses in monkey kidney tissue culture." Proc Soc Exp Biol Med **123**(1): 238-41.

Douglas, R. G. J., Lindgren, K.M., Couch, R.B. (1968). "Exposure to cold environment and rhinovirus common cold: Failure to demonstrate exffect." N Engl J Med **279**: 742-747.

Douglas, R. G., Jr. (1970). "Pathogenesis of rhinovirus common colds in human voluteers." Ann Otol Rhinol Laryngol **79**(3): 563-71.

Douglas, R. M., Moore, B. W., Miles, H. B., Davies, L. M., Graham, N. M., Ryan, P., Worswick, D. A. and Albrecht, J. K. (1986). "Prophylactic efficacy of intranasal alpha 2-interferon against rhinovirus infections in the family setting." N Engl J Med **314**(2): 65-70.

Drake, J. W. and Holland, J. J. (1999). "Mutation rates among RNA viruses." Proc Natl Acad Sci U S A **96**(24): 13910-3.

Duechler, M., Skern, T., Sommergruber, W., Neubauer, C., Gruendler, P., Fogy, I., Blaas, D. and Kuechler, E. (1987). "Evolutionary relationships within the human rhinovirus genus: comparison of serotypes 89, 2, and 14." Proc Natl Acad Sci U S A **84**(9): 2605-9.

Dussart, P., Cartet, G., Huguet, P., Leveque, N., Hajjar, C., Morvan, J., Vanderkerckhove, J., Ferret, K., Lina, B., Chomel, J. J. and Norder, H. (2005). "Outbreak of acute hemorrhagic conjunctivitis in French Guiana and West Indies caused by coxsackievirus A24 variant: phylogenetic analysis reveals Asian import." J Med Virol **75**(4): 559-65.

Farr, B. M., Gwaltney, J. M., Jr., Hendley, J. O., Hayden, F. G., Naclerio, R. M., McBride, T., Doyle, W. J., Sorrentino, J. V., Riker, D. K. and Proud, D. (1990). "A randomized controlled trial of glucocorticoid prophylaxis against experimental rhinovirus infection." J Infect Dis **162**(5): 1173-7.

Felsenstein, J. (1985). "Confidence limits on phylogenies: An approach using the bootstrap." Evolution **39**: 783-791.

Felsenstein, J. (2004). Inferring phylogenies. Sunderland, Sinauer Associates, Inc.

Felsenstein, J. (2005) "PHYLIP (Phylogeny Inference Package) and documentation 3.65, Department of Genetics, University of Washington, Seattle."

Feng, D. F. and Doolittle, R. F. (1996). "Progressive alignment of amino acid sequences and construction of phylogenetic trees from them." Methods Enzymol **266**: 368-82.

Gaffey, M. J., Gwaltney, J. M., Jr., Dressler, W. E., Sorrentino, J. V. and Hayden, F. G. (1987a). "Intranasally administered atropine methonitrate treatment of experimental rhinovirus colds." Am Rev Respir Dis **135**(1): 241-4.

Gaffey, M. J., Gwaltney, J. M., Jr., Sastre, A., Dressler, W. E., Sorrentino, J. V. and Hayden, F. G. (1987b). "Intranasally and orally administered antihistamine treatment of experimental rhinovirus colds." Am Rev Respir Dis **136**(3): 556-60.

Gaffey, M. J., Hayden, F. G., Boyd, J. C. and Gwaltney, J. M., Jr. (1988a). "Ipratropium bromide treatment of experimental rhinovirus infection." Antimicrob Agents Chemother **32**(11): 1644-7.

Gaffey, M. J., Kaiser, D. L. and Hayden, F. G. (1988b). "Ineffectiveness of oral terfenadine in natural colds: evidence against histamine as a mediator of common cold symptoms." Pediatr Infect Dis J **7**(3): 223-8.

Gama, R. E., Hughes, P. J., Bruce, C. B. and Stanway, G. (1988). "Polymerase chain reaction amplification of rhinovirus nucleic acids from clinical material." Nucleic Acids Res **16**(19): 9346.

Gama, R. E., Horsnell, P. R., Hughes, P. J., North, C., Bruce, C. B., al-Nakib, W. and Stanway, G. (1989). "Amplification of rhinovirus specific nucleic acids from clinical samples using the polymerase chain reaction." J Med Virol **28**(2): 73-7.

Geist, F. C., Bateman, J. A. and Hayden, F. G. (1987). "In vitro activity of zinc salts against human rhinoviruses." Antimicrob Agents Chemother **31**(4): 622-4.

Gern, J. E. (2002). "Rhinovirus respiratory infections and asthma." Am J Med **112 Suppl 6A**: 19S-27S.

Ghosh, S., Champlin, R., Couch, R., Englund, J., Raad, I., Malik, S., Luna, M. and Whimbey, E. (1999). "Rhinovirus infections in myelosuppressed adult blood and marrow transplant recipients." Clin Infect Dis **29**(3): 528-32.

Giranda, V. L., Heinz, B. A., Oliveira, M. A., Minor, I., Kim, K. H., Kolatkar, P. R., Rossmann, M. G. and Rueckert, R. R. (1992). "Acid-induced structural changes in human rhinovirus 14: possible role in uncoating." Proc Natl Acad Sci U S A **89**(21): 10213-7.

Gjoen, K., Bruu, A.-L., and and Orstavik, I. (1995). "Intratypic genome variability of echovirus type 30 in part of the VP4/VP2 coding region." Arch Virol **141**: 901-908.

Gmyl, A. P., Belousov, E. V., Maslova, S. V., Khitrina, E. V., Chetverin, A. B. and Agol, V. I. (1999). "Nonreplicative RNA recombination in poliovirus." J Virol **73**(11): 8958-65.

Gmyl, A. P., Korshenko, S. A., Belousov, E. V., Khitrina, E. V. and Agol, V. I. (2003). "Nonreplicative homologous RNA recombination: promiscuous joining of RNA pieces?" RNA **9**(10): 1221-31.

Greenberg, S. B. (2002). "Viral respiratory infections in elderly patients and patients with chronic obstructive pulmonary disease." Am J Med **112 Suppl 6A**: 28S-32S.

Greve, J. M., Davis, G., Meyer, A. M., Forte, C. P., Yost, S. C., Marlor, C. W., Kamarck, M. E. and McClelland, A. (1989). "The major human rhinovirus receptor is ICAM-1." Cell **56**(5): 839-47.

Greve, J. M., Forte, C. P., Marlor, C. W., Meyer, A. M., Hoover-Litty, H., Wunderlich, D. and McClelland, A. (1991). "Mechanisms of receptor-mediated rhinovirus neutralization defined by two soluble forms of ICAM-1." J Virol **65**(11): 6015-23.

Gruenberger, M., Wandl, R., Nimpf, J., Hiesberger, T., Schneider, W. J., Kuechler, E. and Blaas, D. (1995). "Avian homologs of the mammalian low-density lipoprotein receptor family bind minor receptor group human rhinovirus." J Virol **69**(11): 7244-7.

Gustafson, L. M., Proud, D., Hendley, J. O., Hayden, F. G. and Gwaltney, J. M., Jr. (1996). "Oral prednisone therapy in experimental rhinovirus infections." J Allergy Clin Immunol **97**(4): 1009-14.

Gwaltney, J. M., Jr., Hendley, J. O., Simon, G. and Jordan, W. S., Jr. (1966). "Rhinovirus infections in an industrial population. I. The occurrence of illness." N Engl J Med **275**(23): 1261-8.

Gwaltney, J. M., Jr., Moskalski, P. B. and Hendley, J. O. (1978). "Hand-to-hand transmission of rhinovirus colds." Ann Intern Med **88**(4): 463-7.

Gwaltney, J. M., Jr., Park, J., Paul, R. A., Edelman, D. A., O'Connor, R. R. and Turner, R. B. (1996). "Randomized controlled trial of clemastine fumarate for treatment of experimental rhinovirus colds." Clin Infect Dis **22**(4): 656-62.

Gwaltney, J. M. (2002a). "Clinical significance and pathogenesis of viral respiratory infections." Am J Med **112 Suppl 6A**: 13S-18S.

Gwaltney, J. M. (2002b). "Viral respiratory infection therapy: historical perspectives and current trials." Am J Med **112 Suppl 6A**: 33S-41S.

Halonen, P., Rocha, E., Hierholzer, J., Holloway, B., Hyypiä, T., Hurskainen, P. and Pallansch, M. (1995). "Detection of enteroviruses and rhinoviruses in clinical specimens by PCR and liquid-phase hybridization." J Clin Microbiol **33**(3): 648-53.

Hamparian, V. V., Colonno, R. J., Cooney, M. K., Dick, E. C., Gwaltney, J. M., Jr., Hughes, J. H., Jordan, W. S., Jr., Kapikian, A. Z., Mogabgab, W. J., Monto, A. and et al. (1987). "A

collaborative report: rhinoviruses--extension of the numbering system from 89 to 100." Virology **159**(1): 191-2.

Harris, J. R. and Racaniello, V. R. (2005). "Amino acid changes in proteins 2B and 3A mediate rhinovirus type 39 growth in mouse cells." J Virol **79**(9): 5363-73.

Hasegawa, M., Kishino, H. and Yano, T. (1985). "Dating of the human-ape splitting by a molecular clock of mitochondrial DNA." J Mol Evol **22**(2): 160-74.

Hayden, F. G. and Gwaltney, J. M., Jr. (1984). "Intranasal interferon-alpha 2 treatment of experimental rhinoviral colds." J Infect Dis **150**(2): 174-80.

Hayden, F. G., Herrington, D. T., Coats, T. L., Kim, K., Cooper, E. C., Villano, S. A., Liu, S., Hudson, S., Pevear, D. C., Collett, M. and McKinlay, M. (2003a). "Efficacy and safety of oral pleconaril for treatment of colds due to picornaviruses in adults: results of 2 double-blind, randomized, placebo-controlled trials." Clin Infect Dis **36**(12): 1523-32.

Hayden, F. G., Turner, R. B., Gwaltney, J. M., Chi-Burris, K., Gersten, M., Hsyu, P., Patick, A. K., Smith, G. J., 3rd and Zalman, L. S. (2003b). "Phase II, randomized, double-blind, placebo-controlled studies of ruprintrivir nasal spray 2-percent suspension for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers." Antimicrob Agents Chemother **47**(12): 3907-16.

Hayden, F. G. (2004). "Rhinovirus and the lower respiratory tract." Rev Med Virol **14**(1): 17-31.

Hendley, J. O., Wenzel, R. P. and Gwaltney, J. M., Jr. (1973). "Transmission of rhinovirus colds by self-inoculation." N Engl J Med **288**(26): 1361-4.

Hewat, E. A., Neumann, E., Conway, J. F., Moser, R., Ronacher, B., Marlovits, T. C. and Blaas, D. (2000). "The cellular receptor to human rhinovirus 2 binds around the 5-fold axis and not in the canyon: a structural view." EMBO J **19**(23): 6317-25.

Hilleman, M. R. (1967). "Present knowledge of the rhinovirus group of viruses." Curr Top Microbiol Immunol **41**: 1-22.

Hillis, D. M. a. J. J. B. (1993). "An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis." Syst. Biol. **42**: 182-192.

Hirst, G. K. (1962). "Genetic recombination with Newcastle disease virus, polioviruses, and influenza." Cold Spring Harb Symp Quant Biol **27**: 303-9.

Hofer, F., Gruenberger, M., Kowalski, H., Machat, H., Huettinger, M., Kuechler, E. and Blass, D. (1994). "Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus." Proc Natl Acad Sci U S A **91**(5): 1839-42.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. and VandePol, S. (1982). "Rapid evolution of RNA genomes." Science **215**(4540): 1577-85.

Holland, J. J., De La Torre, J. C. and Steinhauer, D. A. (1992). "RNA virus populations as quasispecies." Curr Top Microbiol Immunol **176**: 1-20.

Holland, J. and Domingo, E. (1998). "Origin and evolution of viruses." Virus Genes **16**(1): 13-21.

Horsnell, C., Gama, R. E., Hughes, P. J. and Stanway, G. (1995). "Molecular relationships between 21 human rhinovirus serotypes." J Gen Virol **76 (Pt 10)**: 2549-55.

Hovi, T., Stenvik, M. and Rosenlew, M. (1996). "Relative abundance of enterovirus serotypes in sewage differs from that in patients: clinical and epidemiological implications." Epidemiol Infect **116**(1): 91-7.

Hsia, J., Simon, G. L., Higgins, N., Goldstein, A. L. and Hayden, F. G. (1989). "Immune modulation by aspirin during experimental rhinovirus colds." Bull N Y Acad Med **65**(1): 45-56.

Hughes, J. H., Thomas, D. C., Hamparian, V. V. and Cramblett, H. G. (1973). "Acid liability of rhinovirus type 14: effect of pH, time, and temperature." Proc Soc Exp Biol Med **144**(2): 555-60.

Hughes, P. J., North, C., Jellis, C. H., Minor, P. D. and Stanway, G. (1988). "The nucleotide sequence of human rhinovirus 1B: molecular relationships within the rhinovirus genus." J Gen Virol **69 (Pt 1)**: 49-58.

Hyöty, H., Hiltunen, M., Knip, M., Laakkonen, M., Vahasalo, P., Karjalainen, J., Koskela, P., Roivainen, M., Leinikki, P., Hovi, T. and et al. (1995). "A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group." Diabetes **44**(6): 652-7.

Hyypiä, T., Auvinen, P. and Maaronen, M. (1989). "Polymerase chain reaction for human picornaviruses." J Gen Virol **70 (Pt 12)**: 3261-8.

Hyypiä, T., Puhakka, T., Ruuskanen, O., Mäkelä, M., Arola, A. and Arstila, P. (1998). "Molecular diagnosis of human rhinovirus infections: comparison with virus isolation." J Clin Microbiol **36**(7): 2081-3.

International Molecular Epidemiology Task Force. (1998). "What is Molecular Epidemiology?" The International Molecular Epidemiology Task Force (IMETAF). Retrieved 24.10.2005, from http://www.pitt.edu/~kkr/molepi.html.

Ireland, D. C., Kent, J. and Nicholson, K. G. (1993). "Improved detection of rhinoviruses in nasal and throat swabs by seminested RT-PCR." J Med Virol **40**(2): 96-101.

Ishiko, H., Miura, R., Shimada, Y., Hayashi, A., Nakajima, H., Yamazaki, S. and Takeda, N. (2002). "Human rhinovirus 87 identified as human enterovirus 68 by VP4-based molecular diagnosis." Intervirology **45**(3): 136-41.

Jartti, T., Lehtinen, P., Vuorinen, T., Koskenvuo, M. and Ruuskanen, O. (2004). "Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children." J Med Virol **72**(4): 695-9.

Jenison, R., Rihanek, M. and Polisky, B. (2001). "Use of a thin film biosensor for rapid visual detection of PCR products in a multiplex format." Biosens Bioelectron **16**(9-12): 757-63.

Jennings, L. C. and Dick, E. C. (1987). "Transmission and control of rhinovirus colds." Eur J Epidemiol **3**(4): 327-35.

Johnston, S. L., Sanderson, G., Pattemore, P. K., Smith, S., Bardin, P. G., Bruce, C. B., Lambden, P. R., Tyrrell, D. A. and Holgate, S. T. (1993). "Use of polymerase chain reaction for diagnosis of picornavirus infection in subjects with and without respiratory symptoms." J Clin Microbiol **31**(1): 111-7.

Johnston, S. L., Pattemore, P. K., Sanderson, G., Smith, S., Lampe, F., Josephs, L., Symington, P., O'Toole, S., Myint, S. H., Tyrrell, D. A. and et al. (1995). "Community study of role of viral infections in exacerbations of asthma in 9-11 year old children." BMJ **310**(6989): 1225-9.

Kaiser, L., Crump, C. E. and Hayden, F. G. (2000). "In vitro activity of pleconaril and AG7088 against selected serotypes and clinical isolates of human rhinoviruses." Antiviral Res **47**(3): 215-20.

Kapikian, A. Z., (Chairman), , Conant, R. M., Hamparian, V. V., Chanock, R. M., Chapple, P. J., Dick, E. C., Fenters, J. D., Gwaltney, J. M., Jr., , Hamre, D., Holper, J. C., Jordan, W. S., Lennette, E. H., Melnick, J. L., Mogabgab, W. J., Mufson, M. A. and Phillips, C. A. (1967). "Rhinoviruses: a numbering system." Nature (London) **213**: 761-763.

Kapikian, A. Z. C., Conant, R. M., Hamparian, V. V., Chanock, R. M., Dick, E. C., Gwaltney, J. M., Jr., , Hamre, D., Jordan, W. S., Jr., , Kenny, K. E., Lennette, E. H., Melnick, J. L., Mogabgab, W. J., Phillips, C. A., Schieble, J. H., Stott, E. J., and and Tyrrell, D. A. J. (1971). "A collaborative report: Rhinoviruses - extension of the numbering system." Virology **43**: 524-526.

Kares, S., Lonnrot, M., Vuorinen, P., Oikarinen, S., Taurianen, S. and Hyoty, H. (2004). "Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler." J Clin Virol **29**(2): 99-104.

Kew, O. M., Mulders, M.N., Lipskaya, G.Y., da Silva, E.E., and Pallansch, M.A. (1995). "Molecular epidemiology of polioviruses." Seminars in Virology **6**: 401-414.

Kew, O., Morris-Glasgow, V., Landaverde, M., Burns, C., Shaw, J., Garib, Z., Andre, J., Blackman, E., Freeman, C. J., Jorba, J., Sutter, R., Tambini, G., Venczel, L., Pedreira, C., Laender, F., Shimizu, H., Yoneyama, T., Miyamura, T., van Der Avoort, H., Oberste, M. S., Kilpatrick, D., Cochi, S., Pallansch, M. and de Quadros, C. (2002). "Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus." Science **296**(5566): 356-9.

Kew, O. M., Sutter, R. W., de Gourville, E. M., Dowdle, W. R. and Pallansch, M. A. (2005). "Vaccine-Derived Polioviruses and the Endgame Strategy for Global Polio Eradication." Annu Rev Microbiol **59**: 587-635.

Kilpatrick, D. R., Quay, J., Pallansch, M. A. and Oberste, M. S. (2001). "Type-specific detection of echovirus 30 isolates using degenerate reverse transcriptase PCR primers." J Clin Microbiol **39**(4): 1299-302.

Kinnunen, L., Huovilainen, A., Pöyry, T. and Hovi, T. (1990). "Rapid molecular evolution of wild type 3 poliovirus during infection in individual hosts." J Gen Virol **71 (Pt 2)**: 317-24.

Kinnunen, L., Pöyry, T. and Hovi, T. (1991). "Generation of virus genetic lineages during an outbreak of poliomyelitis." J Gen Virol **72 (Pt 10)**: 2483-9.

Kolatkar, P. R., Bella, J., Olson, N. H., Bator, C. M., Baker, T. S. and Rossmann, M. G. (1999). "Structural studies of two rhinovirus serotypes complexed with fragments of their cellular receptor." EMBO J **18**(22): 6249-59.

Kopecka, H., Brown, B. and Pallansch, M. (1995). "Genotypic variation in coxsackievirus B5 isolates from three different outbreaks in the United States." Virus Res **38**(2-3): 125-36.

Kuhner, M. K. and Felsenstein, J. (1994). "A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates." Mol Biol Evol **11**(3): 459-68.

Kunkel, U. and Schreier, E. (2000). "Genetic variability within the VP1 coding region of echovirus type 30 isolates." Arch Virol **145**(7): 1455-64.

Laine, P., Savolainen, C., Blomqvist, S. and Hovi, T. (2005). "Phylogenetic analysis of human rhinovirus capsid protein VP1 and 2A protease coding sequences confirms shared genus-like relationships with human enteroviruses." J Gen Virol **86**(Pt 3): 697-706.

Las Heras, J. and Swanson, V. L. (1983). "Sudden death of an infant with rhinovirus infection complicating bronchial asthma: case report." Pediatr Pathol **1**(3): 319-23.

Leckie, G. W. (1987). Molecular studies on a human common cold virus, Human rhinovirus nine., University of Leicester.

Ledford, R. M., Patel, N. R., Demenczuk, T. M., Watanyar, A., Herbertz, T., Collett, M. S. and Pevear, D. C. (2004). "VP1 sequencing of all human rhinovirus serotypes: insights into genus phylogeny and susceptibility to antiviral capsid-binding compounds." J Virol **78**(7): 3663-74.

Ledinko, N. (1963). "Genetic recombination with poliovirus type 1. Studies of crosses between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain." Virology **20**: 107-19.

Lee, W. M., Wang, W. and Rueckert, R. R. (1995). "Complete sequence of the RNA genome of human rhinovirus 16, a clinically useful common cold virus belonging to the ICAM-1 receptor group." Virus Genes **9**(2): 177-81.

Lindberg, A. M., Andersson, P., Savolainen, C., Mulders, M. N. and Hovi, T. (2003). "Evolution of the genome of Human enterovirus B: incongruence between phylogenies of the VP1 and 3CD regions indicates frequent recombination within the species." J Gen Virol **84**(Pt 5): 1223-35.

Lipskaya, G., Chervonskaya, E. A., Belova, G. I., Maslova, S. V., Kutateladze, T. N., Drozdov, S. G., Mulders, M., Pallansch, M. A., Kew, O. M. and Agol, V. I. (1995). "Geographical genotypes (geotypes) of poliovirus case isolates from the former Soviet Union: relatedness to other known poliovirus genotypes." J Gen Virol **76 (Pt 7)**: 1687-99.

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W. and Ray, S. C. (1999). "Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination." J Virol **73**(1): 152-60.

Lönnrot, M., Sjöroos, M., Salminen, K., Maaronen, M., Hyypiä, T. and Hyöty, H. (1999). "Diagnosis of enterovirus and rhinovirus infections by RT-PCR and time-resolved fluorometry with lanthanide chelate labeled probes." J Med Virol **59**(3): 378-84.

Lukashev, A. N., Lashkevich, V. A., Koroleva, G. A. and Karganova, G. G. (2002). "Phylogenetic and serological characterization of echovirus 11 and echovirus 19 strains causing uveitis." Arch Virol **147**(1): 131-42.

Lukashev, A. N., Lashkevich, V. A., Ivanova, O. E., Koroleva, G. A., Hinkkanen, A. E. and Ilonen, J. (2003a). "Recombination in circulating enteroviruses." J Virol **77**(19): 10423-31.

Lukashev, A. N., Lashkevich, V. A., Koroleva, G. A., Ilonen, J., Karganova, G. G., Reznik, V. I. and Hinkkanen, A. E. (2003b). "Molecular epidemiology of enteroviruses causing uveitis and multisystem hemorrhagic disease of infants." Virology **307**(1): 45-53.

Lukashev, A. N., Lashkevich, V. A., Koroleva, G. A., Ilonen, J. and Hinkkanen, A. E. (2004). "Recombination in uveitis-causing enterovirus strains." J Gen Virol **85**(Pt 2): 463-70.

Lukashev, A. N. (2005). "Role of recombination in evolution of enteroviruses." Rev Med Virol **15**(3): 157-67.

Maguire, H. C., Atkinson, P., Sharland, M. and Bendig, J. (1999). "Enterovirus infections in England and Wales: laboratory surveillance data: 1975 to 1994." Commun Dis Public Health **2**(2): 122-5.

Mäkelä, M. J., Puhakka, T., Ruuskanen, O., Leinonen, M., Saikku, P., Kimpimäki, M., Blomqvist, S., Hyypiä, T. and Arstila, P. (1998). "Viruses and bacteria in the etiology of the common cold." J Clin Microbiol **36**(2): 539-42.

Manzara, S., Muscillo, M., La Rosa, G., Marianelli, C., Cattani, P. and Fadda, G. (2002). "Molecular identification and typing of enteroviruses isolated from clinical specimens." J Clin Microbiol **40**(12): 4554-60.

Marlovits, T. C., Abrahamsberg, C. and Blaas, D. (1998). "Very-low-density lipoprotein receptor fragment shed from HeLa cells inhibits human rhinovirus infection." J Virol **72**(12): 10246-50.

Martin, J., Samoilovich, E., Dunn, G., Lackenby, A., Feldman, E., Heath, A., Svirchevskaya, E., Cooper, G., Yermalovich, M. and Minor, P. D. (2002). "Isolation of an intertypic poliovirus capsid recombinant from a child with vaccine-associated paralytic poliomyelitis." J Virol **76**(21): 10921-8.

Melnick, J. L. (1996). Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. Fields Virology, 3rd ed. B. Fields, Knipe, DM, Howley, PM et al. Philadelphia, Lippincott-Raven**:** 655-712.

Microbiology @ Leicester. (2004). "Glossary of virology." Microbiology @ Leicester. Retrieved 24.10.2005, from http://www-micro.msb.le.ac.uk/MBChB/VirGloss.html.

Mogabgab, W. J., Holmes, B. J. and Pollock, B. (1975). "Antigenic relationships of common rhinovirus types from disabling upper respiratory illnesses." Dev Biol Stand **28**: 400-11.

Mori, J. and Clewley, J. P. (1994). "Polymerase chain reaction and sequencing for typing rhinovirus RNA." J Med Virol **44**(4): 323-9.

Mori, I., Matsumoto, K., Hatano, M., Sudo, M. and Kimura, Y. (1995). "An unseasonable winter outbreak of echovirus type 30 meningitis." J Infect **31**(3): 219-23.

Mufson, M. A., Kawana, R., James, H. D., Jr., Gauld, L. W., Bloom, H. H. and Chanock, R. M. (1965). "A Description of Six New Rhinoviruses of Human Origin." Am J Epidemiol **81**: 32-43.

Mulders, M. N., Lipskaya, G. Y., van der Avoort, H. G., Koopmans, M. P., Kew, O. M. and van Loon, A. M. (1995a). "Molecular epidemiology of wild poliovirus type 1 in Europe, the Middle East, and the Indian subcontinent." J Infect Dis **171**(6): 1399-405.

Mulders, M. N., van Loon, A. M., van der Avoort, H. G., Reimerink, J. H., Ras, A., Bestebroer, T. M., Drebot, M. A., Kew, O. M. and Koopmans, M. P. (1995b). "Molecular characterization of a wild poliovirus type 3 epidemic in The Netherlands (1992 and 1993)." J Clin Microbiol **33**(12): 3252-6.

Mulders, M. N., Reimerink, J. H., Koopmans, M. P., van Loon, A. M. and van der Avoort, H. G. (1997). "Genetic analysis of wild-type poliovirus importation into The Netherlands (1979-1995)." J Infect Dis **176**(3): 617-24.

Mulders, M. N., Salminen, M., Kalkkinen, N. and Hovi, T. (2000). "Molecular epidemiology of coxsackievirus B4 and disclosure of the correct VP1/2A(pro) cleavage site: evidence for high genomic diversity and long-term endemicity of distinct genotypes." J Gen Virol **81**(Pt 3): 803-12.

Mullins, J. A., Khetsuriani, N., Nix, W. A., Oberste, M. S., LaMonte, A., Kilpatrick, D. R., Dunn, J., Langer, J., McMinn, P., Huang, Q. S., Grimwood, K., Huang, C. and Pallansch, M. A. (2004). "Emergence of echovirus type 13 as a prominent enterovirus." Clin Infect Dis **38**(1): 70-7.

Naclerio, R. M., Proud, D., Lichtenstein, L. M., Kagey-Sobotka, A., Hendley, J. O., Sorrentino, J. and Gwaltney, J. M. (1988). "Kinins are generated during experimental rhinovirus colds." J Infect Dis **157**(1): 133-42.

Nairn, C. and Clements, G. B. (1999). "A study of enterovirus isolations in Glasgow from 1977 to 1997." J Med Virol **58**(3): 304-12.

National Cancer Institute. (2005). "Glossary." National Cancer Institute. Retrieved 24.10.2005, from http://plan2005.cancer.gov/glossary.html.

Neubauer, C., Frasel, L., Kuechler, E. and Blaas, D. (1987). "Mechanism of entry of human rhinovirus 2 into HeLa cells." Virology **158**(1): 255-8.

Noah, T. L., Henderson, F. W., Wortman, I. A., Devlin, R. B., Handy, J., Koren, H. S. and Becker, S. (1995). "Nasal cytokine production in viral acute upper respiratory infection of childhood." J Infect Dis **171**(3): 584-92.

Nokso-Koivisto, J., Räty, R., Blomqvist, S., Kleemola, M., Syrjänen, R., Pitkäranta, A., Kilpi, T. and Hovi, T. (2004). "Presence of specific viruses in the middle ear fluids and respiratory secretions of young children with acute otitis media." J Med Virol **72**(2): 241-8.

Norder, H., Bjerregaard, L. and Magnius, L. O. (2001). "Homotypic echoviruses share aminoterminal VP1 sequence homology applicable for typing." J Med Virol **63**(1): 35-44.

Oberste, M. S., Maher, K. and Pallansch, M. A. (1998). "Molecular phylogeny of all human enterovirus serotypes based on comparison of sequences at the 5' end of the region encoding VP2." Virus Res **58**(1-2): 35-43.

Oberste, M. S., Maher, K., Kennett, M. L., Campbell, J. J., Carpenter, M. S., Schnurr, D. and Pallansch, M. A. (1999a). "Molecular epidemiology and genetic diversity of echovirus type 30 (E30): genotypes correlate with temporal dynamics of E30 isolation." J Clin Microbiol **37**(12): 3928-33.

Oberste, M. S., Maher, K., Kilpatrick, D. R., Flemister, M. R., Brown, B. A. and Pallansch, M. A. (1999b). "Typing of human enteroviruses by partial sequencing of VP1." J Clin Microbiol **37**(5): 1288-93.

Oberste, M. S., Maher, K., Kilpatrick, D. R. and Pallansch, M. A. (1999c). "Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification." J Virol **73**(3): 1941-8.

Oberste, M. S., Maher, K., Flemister, M. R., Marchetti, G., Kilpatrick, D. R. and Pallansch, M. A. (2000). "Comparison of classic and molecular approaches for the identification of untypeable enteroviruses." J Clin Microbiol **38**(3): 1170-4.

Oberste, M. S., Nix, W. A., Kilpatrick, D. R., Flemister, M. R. and Pallansch, M. A. (2003a). "Molecular epidemiology and type-specific detection of echovirus 11 isolates from the Americas, Europe, Africa, Australia, southern Asia and the Middle East." Virus Res **91**(2): 241-8.

Oberste, M. S., Nix, W. A., Maher, K. and Pallansch, M. A. (2003b). "Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing." J Clin Virol **26**(3): 375-7.

Oberste, M. S., Maher, K. and Pallansch, M. A. (2004a). "Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes." J Virol **78**(2): 855-67.

Oberste, M. S., Maher, K., Schnurr, D., Flemister, M. R., Lovchik, J. C., Peters, H., Sessions, W., Kirk, C., Chatterjee, N., Fuller, S., Hanauer, J. M. and Pallansch, M. A. (2004b). "Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses." J Gen Virol **85**(Pt 9): 2577-84.

Olive, D. M., Al-Mufti, S., Al-Mulla, W., Khan, M. A., Pasca, A., Stanway, G. and Al-Nakib, W. (1990). "Detection and differentiation of picornaviruses in clinical samples following genomic amplification." J Gen Virol **71 (Pt 9)**: 2141-7.

Olson, N. H., Kolatkar, P. R., Oliveira, M. A., Cheng, R. H., Greve, J. M., McClelland, A., Baker, T. S. and Rossmann, M. G. (1993). "Structure of a human rhinovirus complexed with its receptor molecule." Proc Natl Acad Sci U S A **90**(2): 507-11.

Oprisan, G., Combiescu, M., Guillot, S., Caro, V., Combiescu, A., Delpeyroux, F. and Crainic, R. (2002). "Natural genetic recombination between co-circulating heterotypic enteroviruses." J Gen Virol **83**(Pt 9): 2193-200.

Ozkaya, E., Hizel, K., Uysal, G., Akman, S., Terzioglu, S. and Kuyucu, N. (2003). "An outbreak of Aseptic Meningitis due to echovirus type 30 in two cities of Turkey." Eur J Epidemiol **18**(8): 823-6.

Page, R. D. (1996). "TreeView: an application to display phylogenetic trees on personal computers." Comput Appl Biosci **12**(4): 357-8.

Palacios, G., Casas, I., Cisterna, D., Trallero, G., Tenorio, A. and Freire, C. (2002a). "Molecular epidemiology of echovirus 30: temporal circulation and prevalence of single lineages." J Virol **76**(10): 4940-9.

Palacios, G., Casas, I., Tenorio, A. and Freire, C. (2002b). "Molecular identification of enterovirus by analyzing a partial VP1 genomic region with different methods." J Clin Microbiol **40**(1): 182-92.

Palmenberg, A. C. (1989). Sequence alignments of picornaviral capsid proteins. Molecular aspects of picornavirus infection and detection. Washington, DC, American Society of Microbiology**:** 211-241.

Papadopoulos, N. G., Sanderson, G., Hunter, J. and Johnston, S. L. (1999). "Rhinoviruses replicate effectively at lower airway temperatures." J Med Virol **58**(1): 100-4.

Papadopoulos, N. G., Bates, P. J., Bardin, P. G., Papi, A., Leir, S. H., Fraenkel, D. J., Meyer, J., Lackie, P. M., Sanderson, G., Holgate, S. T. and Johnston, S. L. (2000). "Rhinoviruses infect the lower airways." J Infect Dis **181**(6): 1875-84.

Perriere, G., and Gouy, M. (1996). "WWW-query: an on-line retrieval system for biological sequence banks." Biochimie **78**: 364-369.

Phillips, C. A., Riggs, S., Melnick, J. L. and Grim, C. A. (1965). "Rhinoviruses Associated with Common Colds in a Student Population." JAMA **192**: 277-80.

Pitkäranta, A., Arruda, E., Malmberg, H. and Hayden, F. G. (1997). "Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR." J Clin Microbiol **35**(7): 1791-3.

Pitkäranta, A., Virolainen, A., Jero, J., Arruda, E. and Hayden, F. G. (1998). "Detection of rhinovirus, respiratory syncytial virus, and coronavirus infections in acute otitis media by reverse transcriptase polymerase chain reaction." Pediatrics **102**(2 Pt 1): 291-5.

Pitkäranta, A., Starck, M., Savolainen, S., Pöyry, T., Suomalainen, I., Hyypiä, T., Carpen, O. and Vaheri, A. (2001). "Rhinovirus RNA in the maxillary sinus epithelium of adult patients with acute sinusitis." Clin Infect Dis **33**(6): 909-11.

Pöyry, T., Kinnunen, L., Kapsenberg, J., Kew, O. and Hovi, T. (1990). "Type 3 poliovirus/Finland/1984 is genetically related to common Mediterranean strains." J Gen Virol **71 (Pt 11)**: 2535-41.

Prchla, E., Kuechler, E., Blaas, D. and Fuchs, R. (1994). "Uncoating of human rhinovirus serotype 2 from late endosomes." J Virol **68**(6): 3713-23.

Puhakka, T., Mäkelä, M. J., Alanen, A., Kallio, T., Korsoff, L., Arstila, P., Leinonen, M., Pulkkinen, M., Suonpää, J., Mertsola, J. and Ruuskanen, O. (1998). "Sinusitis in the common cold." J Allergy Clin Immunol **102**(3): 403-8.

Racaniello, V. R. (2001). Picornaviridae: The Viruses and Their Replication. Fields Virology. 4th ed. D. M. Knipe, and Howley, P.M. Philadelphia, Lippincott Williams & Wilkins**:** 685-722.

Reagan, K. J., McGeady, M. L. and Crowell, R. L. (1981). "Persistence of human rhinovirus infectivity under diverse environmental conditions." Appl Environ Microbiol **41**(3): 618-20.

Rico-Hesse, R., Pallansch, M. A., Nottay, B. K. and Kew, O. M. (1987). "Geographic distribution of wild poliovirus type 1 genotypes." Virology **160**(2): 311-22.

Roivainen, M., Ylipaasto, P., Savolainen, C., Galama, J., Hovi, T. and Otonkoski, T. (2002). "Functional impairment and killing of human beta cells by enteroviruses: the capacity is shared by a wide range of serotypes, but the extent is a characteristic of individual virus strains." Diabetologia **45**(5): 693-702.

Rotbart, H. A. (1990). "Enzymatic RNA amplification of the enteroviruses." J Clin Microbiol **28**(3): 438-42.

Saitou, N. and Nei, M. (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees." Mol Biol Evol **4**(4): 406-25.

Sala, M. and Wain-Hobson, S. (2000). "Are RNA viruses adapting or merely changing?" J Mol Evol **51**(1): 12-20.

Salminen, M. O., Carr, J. K., Burke, D. S. and McCutchan, F. E. (1995). "Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning." AIDS Res Hum Retroviruses **11**(11): 1423-5.

Santti, J., Hyypiä, T. and Halonen, P. (1997). "Comparison of PCR primer pairs in the detection of human rhinoviruses in nasopharyngeal aspirates." J Virol Methods **66**(1): 139-47.

Santti, J., Hyypiä, T., Kinnunen, L. and Salminen, M. (1999). "Evidence of recombination among enteroviruses." J Virol **73**(10): 8741-9.

Santti, J., Harvala, H., Kinnunen, L. and Hyypiä, T. (2000). "Molecular epidemiology and evolution of coxsackievirus A9." J Gen Virol **81**(Pt 5): 1361-72.

Schieble, J. H., Fox, V. L., Lester, F. and Lennette, E. H. (1974). "Rhinoviruses: an antigenic study of the prototype virus strains." Proc Soc Exp Biol Med **147**(2): 541-5.

Schierup, M. H. and Hein, J. (2000). "Consequences of recombination on traditional phylogenetic analysis." Genetics **156**(2): 879-91.

Schober, D., Kronenberger, P., Prchla, E., Blaas, D. and Fuchs, R. (1998). "Major and minor receptor group human rhinoviruses penetrate from endosomes by different mechanisms." J Virol **72**(2): 1354-64.

Scholtissek, C. (1996). "Molecular evolution of influenzaviruses." Virus Genes **11**: 209-215.

Schroth, M. K., Grimm, E., Frindt, P., Galagan, D. M., Konno, S. I., Love, R. and Gern, J. E. (1999). "Rhinovirus replication causes RANTES production in primary bronchial epithelial cells." Am J Respir Cell Mol Biol **20**(6): 1220-8.

Schwartz, A. R., Togo, Y., Hornick, R. B., Tominaga, S. and Gleckman, R. A. (1973). "Evaluation of the efficacy of ascorbic acid in prophylaxis of induced rhinovirus 44 infection in man." J Infect Dis **128**(4): 500-5.

Sherry, B., Mosser, A. G., Colonno, R. J. and Rueckert, R. R. (1986). "Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus, human rhinovirus 14." J Virol **57**(1): 246-57.

Sherry, B. and Rueckert, R. (1985). "Evidence for at least two dominant neutralization antigens on human rhinovirus 14." J Virol **53**(1): 137-43.

Skern, T., Sommergruber, W., Blaas, D., Gruendler, P., Fraundorfer, F., Pieler, C., Fogy, I. and Kuechler, E. (1985). "Human rhinovirus 2: complete nucleotide sequence and proteolytic processing signals in the capsid protein region." Nucleic Acids Res **13**(6): 2111-26.

Skern, T., Neubauer, C., Frasel, L., Grundler, P., Sommergruber, W., Zorn, M., Kuechler, E. and Blaas, D. (1987). "A neutralizing epitope on human rhinovirus type 2 includes amino acid residues between 153 and 164 of virus capsid protein VP2." J Gen Virol **68 (Pt 2)**: 315-23.

Skern, T., Torgersen, H., Auer, H., Kuechler, E. and Blaas, D. (1991). "Human rhinovirus mutants resistant to low pH." Virology **183**(2): 757-63.

Smith, T. J. (2001). "Antibody interactions with rhinovirus: lessons for mechanisms of neutralization and the role of immunity in viral evolution." Curr Top Microbiol Immunol **260**: 1-28.

Smyth, A. R., Smyth, RL, Tong, CY, Hart, C. A. and Heaf, D. P. (1995). "Effect of respiratory virus infections including rhinovirus on clinical status in cystic fibrosis." Arch Dis Childhood **73**: 117-120.

Snyers, L., Zwickl, H. and Blaas, D. (2003). "Human rhinovirus type 2 is internalized by clathrin-mediated endocytosis." J Virol **77**(9): 5360-9.

Sokal, R. R., and Mitchener, C.D. (1958). "A statistical method for evaluating systemic relationships." University of Kansas Science Bulletin **38**: 1409-1438.

Sperber, S. J., Levine, P. A., Sorrentino, J. V., Riker, D. K. and Hayden, F. G. (1989). "Ineffectiveness of recombinant interferon-beta serine nasal drops for prophylaxis of natural colds." J Infect Dis **160**(4): 700-5.

Sperber, S. J., Hendley, J. O., Hayden, F. G., Riker, D. K., Sorrentino, J. V. and Gwaltney, J. M., Jr. (1992). "Effects of naproxen on experimental rhinovirus colds. A randomized, double-blind, controlled trial." Ann Intern Med **117**(1): 37-41.

Stanway, G., Hughes, P. J., Mountford, R. C., Minor, P. D. and Almond, J. W. (1984). "The complete nucleotide sequence of a common cold virus: human rhinovirus 14." Nucleic Acids Res **12**(20): 7859-75.

Stanway, G. (1989). "Human rhinovirus 85 (HRV85), complete nucleotide sequence." from http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/index.html.

Stanway, G., Brown, F., Christian, P., Hovi, T., Hyypiä, T., King, A. M. Q., Knowles, N. J., Lemon, S. M., Minor, P. D., Pallansch, M. A., Palmenberg, A. C. a. and Skern, T. (2005). Family Picornaviridae. Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. C. M. Fauquet, Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. London, Elsevier/Academic Press**:** 757-778.

Staunton, D. E., Merluzzi, V. J., Rothlein, R., Barton, R., Marlin, S. D. and Springer, T. A. (1989). "A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses." Cell **56**(5): 849-53.

Steel, M. A., Hendy, M. D. and Penny, D. (1988). "Loss of information in genetic distances." Nature **336**(6195): 118.

Steininger, C., Aberle, S. W. and Popow-Kraupp, T. (2001). "Early detection of acute rhinovirus infections by a rapid reverse transcription-PCR assay." J Clin Microbiol **39**(1): 129-33.

Stott, E. J. and Killington, R. A. (1972). "Rhinoviruses." Annu Rev Microbiol **26**: 503-24.

Strimmer, K., and von Häseler, A. (1996). "Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies." Mol Biol Evol **13**: 964-969.

Strimmer, K. and von Haeseler, A. (1997). "Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment." Proc Natl Acad Sci U S A **94**(13): 6815-9.

Takkouche, B., Regueira, C. and Gestal-Otero, J. J. (2001). "A cohort study of stress and the common cold." Epidemiology **12**(3): 345-9.

Taylor-Robinson, D. and Tyrrell, D. A. (1962). "Serotypes of viruses (rhinoviruses) isolated from common colds." Lancet **1**: 452-4.

Thoelen, I., Lemey, P., Van Der Donck, I., Beuselinck, K., Lindberg, A. M. and Van Ranst, M. (2003). "Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000." J Med Virol **70**(3): 420-9.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids Res **25**(24): 4876-82.

Tomassini, J. E., Graham, D., DeWitt, C. M., Lineberger, D. W., Rodkey, J. A. and Colonno, R. J. (1989). "cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells is intercellular adhesion molecule 1." Proc Natl Acad Sci U S A **86**(13): 4907-11.

Torgersen, H., Skern, T. and Blaas, D. (1989). "Typing of human rhinoviruses based on sequence variations in the 5' non-coding region." J Gen Virol **70 (Pt 11)**: 3111-6.

Turner, R. B., Wecker, M. T., Pohl, G., Witek, T. J., McNally, E., St George, R., Winther, B. and Hayden, F. G. (1999). "Efficacy of tremacamra, a soluble intercellular adhesion molecule 1, for experimental rhinovirus infection: a randomized clinical trial." JAMA **281**(19): 1797-804.

Turner, R. B., Riker, D. K. and Gangemi, J. D. (2000a). "Ineffectiveness of echinacea for prevention of experimental rhinovirus colds." Antimicrob Agents Chemother **44**(6): 1708-9.

Turner, R. B. and Cetnarowski, W. E. (2000b). "Effect of treatment with zinc gluconate or zinc acetate on experimental and natural colds." Clin Infect Dis **31**(5): 1202-8.

Turner, R. B. (2001). "Ineffectiveness of intranasal zinc gluconate for prevention of experimental rhinovirus colds." Clin Infect Dis **33**(11): 1865-70.

Turner, R. B., Biedermann, K. A., Morgan, J. M., Keswick, B., Ertel, K. D. and Barker, M. F. (2004). "Efficacy of organic acids in hand cleansers for prevention of rhinovirus infections." Antimicrob Agents Chemother **48**(7): 2595-8.

Turner, R. B., Bauer, R., Woelkart, K., Hulsey, T. C. and Gangemi, J. D. (2005). "An evaluation of Echinacea angustifolia in experimental rhinovirus infections." N Engl J Med **353**(4): 341-8.

Tyrrell, D. A., and Fielder, M. (2002). The fight against the common cold. New York, Oxford University Press Inc.

Uncapher, C. R., DeWitt, C. M. and Colonno, R. J. (1991). "The major and minor group receptor families contain all but one human rhinovirus serotype." Virology **180**(2): 814-7.

Urquhart, G. E. and Stott, E. J. (1970). "Rhinoviraemia." BMJ **4**(726): 28-30.

Wang, J. R., Tsai, H. P., Huang, S. W., Kuo, P. H., Kiang, D. and Liu, C. C. (2002). "Laboratory diagnosis and genetic analysis of an echovirus 30-associated outbreak of aseptic meningitis in Taiwan in 2001." J Clin Microbiol **40**(12): 4439-44.

Verdaguer, N., Blaas, D. and Fita, I. (2000). "Structure of human rhinovirus serotype 2 (HRV2)." J Mol Biol **300**(5): 1179-94.

Vesa, S., Kleemola, M., Blomqvist, S., Takala, A., Kilpi, T. and Hovi, T. (2001). "Epidemiology of documented viral respiratory infections and acute otitis media in a cohort of children followed from two to twenty-four months of age." Pediatr Infect Dis J **20**(6): 574-81.

Vestergaard, H. T., Johnsen, C. K. and Bottiger, B. (2004). "An unusual enterovirus outbreak in Denmark: clinical characteristics and molecular epidemiology." Scand J Infect Dis **36**(11-12): 840-7.

Whelan, S., Lio, P. and Goldman, N. (2001). "Molecular phylogenetics: state-of-the-art methods for looking into the past." Trends Genet **17**(5): 262-72.

Vlasak, M., Blomqvist, S., Hovi, T., Hewat, E. and Blaas, D. (2003). "Sequence and structure of human rhinoviruses reveal the basis of receptor discrimination." J Virol **77**(12): 6923-30.

Vlasak, M., Roivainen, M., Reithmayer, M., Goesler, I., Laine, P., Snyers, L., Hovi, T. and Blaas, D. (2005). "The minor receptor group of human rhinovirus (HRV) includes HRV23 and HRV25, but the presence of a lysine in the VP1 HI loop is not sufficient for receptor binding." J Virol **79**(12): 7389-95.

World Health Assembly (1988). Global eradication of poliomyelitis by the year 2000. Resolution. WHA41.28. Geneve, World Health Organization.

Xatzipsalti, M., Kyrana, S., Tsolia, M., Psarras, S., Bossios, A., Laza-Stanca, V., Johnston, S. L. and Papadopoulos, N. G. (2005). "Rhinovirus viremia in children with respiratory infections." Am J Respir Crit Care Med **172**(8): 1037-40.

Xiao, C., Tuthill, T. J., Bator Kelly, C. M., Challinor, L. J., Chipman, P. R., Killington, R. A., Rowlands, D. J., Craig, A. and Rossmann, M. G. (2004). "Discrimination among rhinovirus serotypes for a variant ICAM-1 receptor molecule." J Virol **78**(18): 10034-44.

Xing, L., Casasnovas, J. M. and Cheng, R. H. (2003). "Structural analysis of human rhinovirus complexed with ICAM-1 reveals the dynamics of receptor-mediated virus uncoating." J Virol **77**(11): 6101-7.

Ylipaasto, P., Klingel, K., Lindberg, A. M., Otonkoski, T., Kandolf, R., Hovi, T. and Roivainen, M. (2004). "Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells." Diabetologia **47**(2): 225-39.